Pharmacological Studies of *Ilex latifolia* — Hypoglycemic and Hypolipidemic Effects and Lack of Acute Toxicity of *Ilex latifolia* Extract and Its Saponin-enriched Fraction

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Abstract

Today, tea is an important source of beverage. However Chinese also like to mix different plants to make herbal tea for drinking even in the presence of Chinese *Camellia* tea. One of them is Kudingcha, suggesting that Kudingcha may have some medicinal benefits, such as hypolipidemic effect. *Ilex latifolia* (IL) is a Kudingcha, which is rich in saponins. Saponins exert many different biological activities such as anti-allergic activities. Therefore it was worthy to study the medicinal effects of IL extract and a saponin-enriched fraction (SEF) obtained from IL.

In a toxicity study, IL and SEF did not produce liver cell damage after administration for one day and for two weeks. IL and SEF did not induce MROD and EROD activity. Besides SEF had no effect on GST activity either. However IL induced GST activity after administration for one day and two weeks. It appeared that the components in IL for induction of GST activity may not be the saponins.

In a study on the hypoglycemic effect, both IL and SEF reduced the serum glucose level in an oral glucose tolerance experiment. However no hypoglycemic effect was produced in streptozotocin-induced diabetic mice. It is possible that the hypoglycemic effect of IL and SEF is due to a reduced intestinal absorption of glucose. Repeated administrations of IL and SEF reduced the serum glucose level in streptozotocin-induced diabetic mice, suggesting that the hypoglycemic effect of IL

and SEF may be due to an enhancement of glucose utilization besides a reduction in glucose adsorption.

Both IL and SEF reduced serum cholesterol and triglyceride levels in three sets of experiments: mice fed with a high-cholesterol emulsion, hyperlipidemic mice and in the diabetes-induced hyperlipidemic mice. The hypolipidemic effect of IL and SEF may be related to the presence of saponins.

概論

現今，茶已成爲一種重要飲料。雖然飲茶習慣存在已久，但中國人仍喜用不同植物製成藥茶，作爲飲料之用。其中一例爲苦丁茶。由此可知，飲用苦丁茶應有一定的藥效。例如苦丁茶的降脂作用。闊葉冬青(*Ilex latifolia*)爲苦丁茶的一種，並含有豐富的皂甙。而皂甙有很多不同的生物活性，例如抗過敏反應。因此闊葉冬青提取物(IL)及其皂甙成份(SEF)的藥效有其研究價值。

在毒性測試中，飲用 IL 或 SEF 一天及兩星期後，對肝臟沒有嚴重損害，亦對甲氧基試鹵靈 O-脫乙基酶(Methoxyresorufin-O-deethylase)及乙氧基試鹵靈 O-脫乙基酶(Ethoxyresorufin-O-deethylase)沒有任何作用。雖然 SEF 對穀胱甘肽–硫基-轉移酶(Glutathione-S-transferase)沒有誘導作用。但 IL 卻可誘導其活性增加。由此推論，IL 中誘導穀胱甘肽–硫基-轉移酶活性增加的成份可能與皂甙成份無關。

在降血糖實驗中，IL 及 SEF 均可在糖耐量測試中降低小鼠血糖值。但在鏈佐霉素(streptozotocin)誘導糖尿病的小鼠模型中，二者均不能降低其血糖值。由此推斷，其降血糖作用可能與影響小腸糖份吸收有關。但在飲用 IL 及 SEF 一個月後，二者均可降低其血糖值。因此，IL 及 SEF 的效用，除影響吸收糖份外，亦可能與加強體內糖份利用有關。

在小鼠餵食高膽固醇食物實驗，高脂血鼠實驗及糖尿病誘導高脂血小鼠實驗中，IL 及 SEF 均可降低血中膽固醇及甘油三酯量。由此推斷，其降脂作用可能與皂甙成份存在有關。

List of Abbreviations

ACAT	acylCoA: cholesterol acyltransferase
ALT	alanine aminotransferase
AST	aspartate aminotransferase
CHD	coronary heart disease
EROD	ethoxyresorufin O-deethylase
GST	glutathione S-transferase
IL	*Ilex latifolia* extract
MROD	methoxyresorufin O-demethylase
NADP	nicotinamide adenine dinucleotide
$NADP^+$	nicotinamide adenine dinucleotide phosphate
SEF	Saponin-enriched fraction

Chapter 1 Introduction

1.1 History of Tea

1.2 Tea consumption in the world

1.3 Classification of Chinese tea

1.4 Tea and health

1.5 Herbal tea

1.6 Trilex tea

1.7 *Ilex latifolia*

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1.9 Saponins in food and its biological effects

1.10 The saponins in *Ilex latifolia*

1.11 Outline of the thesis

1.1 History of Tea

There is no actual written history supporting the many legends and stories regarding tea's discovery and subsequent development. There were two major legends and it is safe to conclude that the events depicted in the second one may actually be closer to the true events. Emperor Shen Nung of China discovered tea in about 2700 B.C.. Believing that water should be boiled as a hygienic precaution, he discovered a new beverage when tea leaves were blown into boiling water. Being scientifically minded, he tested this new brew and found it refreshing. The second legend is placed in the later Han dynasty, somewhere around 25-221 A.D., where it is mentioned that when Gan Lu returned from his Buddhist studies in India, he brought back tea plants which he then planted in the Meng mountains, in the district of Szechwan. However, the first credible mention was by Liu Kun, a general of the Ching dynasty in the fourth century A.D. who wrote to his nephew, the governor of Yenchow in Shantung province, saying ". . . that he felt aged and depressed and wanted some real t'u" (Palmer, 1999). Then tea production had developed significantly with the transmission of tea seeds and the extension of tea area. More than a thousand year ago, China started to export tea and established trade relationships with over 30 countries in the Middle East, Southeast Asia, West Europe and East Europe (Chen and Lu, 1993).

1.2 Tea consumption in the world

Today, tea is an important source of beverage. In 1994, 2½ million metric tons of tea was produced worldwide. Some of this tea is retained by the producing countries for internal consumption, while the rest is traded either directly to value-added resellers in other countries or bought through auction on the open market. Green tea is consumed primary in China, Japan, and some parts of the Middle East and North Africa, whereas black tea is mostly consumed in Western countries. Tea consumption in selected countries is summarized in Figure 1.1.

1.3 Classification of Chinese tea

There are numerous kinds of tea corresponding with the long history and vast tea areas in China. Chinese tea can be basically divided into six categories, i.e. green tea, black tea, oolong tea, white tea, yellow tea and black-black tea. The classification is based on the degree of fermentation and oxidation of the polyphenols present in the tea leave. The categories of Chinese tea are shown in Table 1.1 (Chen and Lu, 1993).

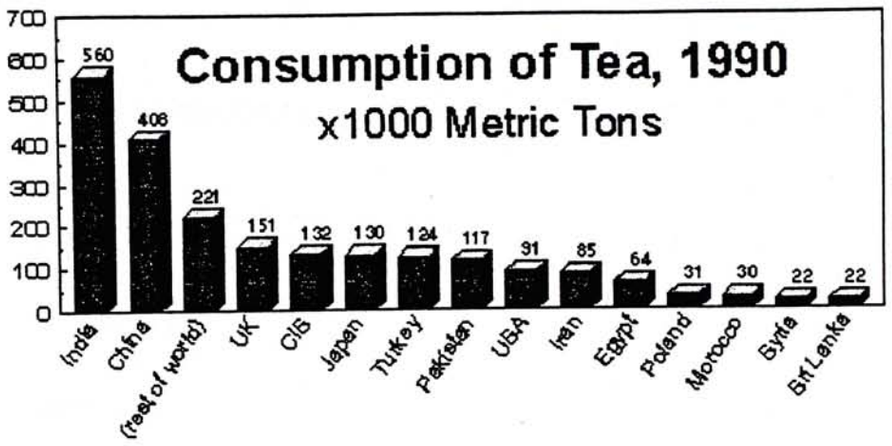

Figure 1.1 Tea consumption in various countries. (Anonymous, 1995)

Table 1.1 Categories of Chinese tea (Chen and Lu, 1995)

Categories	**Features**
Green tea	Non-fermented tea
Black tea	Fermented tea
Oolong tea	Semi-fermented tea
White tea	Light-fermented tea
Yellow tea	Non-enzymetically oxidized tea
Black-black tea	Post-fermented tea

1.4 Tea and health

Tea has been known from the ancient time to possess many medicinal properties but evaluation of its biological activities is still in its infancy. Diverse claims like antidiabetic, antiallergic, antioxidative, antiulcer and antihypertensive properties of tea are sketchy and warrant confirmation (Ganguly, 1993). Most of these studies are limited to Chinese and Japanese green tea extracts containing large amounts of free catechins and low quantities of catechin oxides. Anticarcinogenic, antiatherosclerotic, antimicrobial including anticholeric and anticarious properties of green tea extracts and its polyphenolic constituents are scientifically validated to some extent in animal experiments. The results can partially reflect the beneficial effects of drinking tea.

1.5 Herbal tea

Herbal tea arose out of the climatic conditions of southern China where summers are hot and humid. When people in this region suffered from illness, they sought cost-effective treatments, often turning to local herb-blends. They boiled local plants to address a range of illnesses, including viral infections, liver problems, bone and muscle soreness, and eye infections (Hu, 1997).

In southern China different kinds of hollies are used for herbal tea and medicine. One particular kind of bitter tea, called Kudingcha meaning literally "bitter tea", is

made of the young shoots of *Ilex kudingcha* (Aquifoliaceae). Other closely related species are also used, e.g. *I. latifolia.* (Kong *et al.*, 1998). There are a lot of plants, belonging to different families and genera, which were used as its original materials and are shown in Table 1.2

Kudingcha is extremely bitter as a drink of pleasure. However, connoisseurs still like it even in the presence of Chinese *Camellia* tea. It indicated that partons of Kudingcha must find in it some medicinal properties other than those of tea. Kudingcha and Chinese Holly tea contain more or less the same phytochemical components as *Camellia* tea. Both of them contain triterpene saponins, flavonoids and polyphenols. The beneficial effects of Kudingcha may be related to the presence of similar compounds in *Camellia* tea. The effects of drinking Kudingcha included diuretic, treatment for sore throat, headache and toothache, weight loss, control hypentension, fertility regulation and detoxification (Zhong Yao Da Ci Dian, 1977; But, 1985;Ouyang, 1996).

1.6 Trilex Tea

Trilex tea is a registered brand name owned by The Chinese University of Hong Kong. Its production is very different from that of Kudingcha. It is a product, developed by scientists at The Chinese University of Hong Kong. The aim of the

Table 1.2 The original species of Kudingcha in different areas in China

(Zheng *et al.*, 1992)

Species	Family	Province
Ligustrum pedunculare Rehd	Oleaceae	Sichuan
L. purpurascends Y.C. Yang	Oleaceae	Yunnan
L. japonicum var. pubescens Koidz	Oleaceae	Guizhou
L. robustum (Roxb.)Bl.	Oleaceae	Guizhou
Ilex cornuta Lindl. Ex. Paxt.	Aquifoliaceae	Zhejiang
Ilex kudingcha C. J. Tseng	Aquifoliaceae	Guangxi
Ilex latifolia Thunb.	Aquifoliaceae	Zhejiang, Hunan
Cratoxylum prunifolium (Kurz) Dyer	Guttifera	Guangxi
Ehretia thyrsiflora (S. et Z.) Nakai	Ehretiacae	Guangxi
Photinia serruiata Lindl	Rosaceae	Zhejiang

research was to establish the scientific basis for ethnomedical use of herbal tea used in China (Hu, 1997; Kong and Hu, 1998). Moreover the design of Trilex is based on both experiences of the people and by the principles of multiple complementary ingredients followed in prescription of traditional Chinese medicine. Trilex is a medicated tea with extracts of three hollies and five other crude drugs added to *Camellia* tea or a tea used by farmers in northern China (roasted old mulberry leaves) (Kong *et al.*, 1999). *Ilex latifolia* (IL) is one of the major hollies in Trilex tea and is used for studying its pharmacological effects.

1.7 Ilex latifolia

Holly is a broad-leafed evergreen shrub or tree that may have spiny or smooth leaves and whose berries can be red, orange, yellow or black. Most hollies are either female or male and in order for berries to form (with only a few exceptions), the female holly plant will have to have a male pollinator close by (Lynne, 1999). IL, which is native to China and Japan, has large dull green leaves. A very large holly tree (slow growth to 60 feet) bears large dull red berries. The related information of IL and the photographs of the IL plant and its leaves are shown Figures 1.2 and 1.3. The district distribution of IL covers Anhui, Fujian, Jiangsu and Zhejiang. Besides IL can also be found in Japan. There is little information about the chemical constituents

BOTANICAL NAME: *Ilex latifolia*

COMMON NAME: Lusterleaf Holly

FAMILY: Aquifoliaceae

NATIVITY: China, Japan.

GROWTH HABIT: Pyramidal tree with dense growth.

SIZE: 50 - 60' in its native habitat, 15 - 25' in cultivation.

HARDINESS: Zone 7 - 9.

CULTURE: Full sun, in well-drained, moist soils.

FOLIAGE: Alternate, oblong, with relatively finely serrate margins, 4 - 8" long. Foliage is leathery and course texture. Blends well with the texture and color of Southern magnolia foliage.

FLOWERS: Dioecious, creamy white, quite fragrant.

FRUIT: Drupe, red, about 1/3" long, generally borne in abundance.

Figure 1.2 Information about IL (Mackenzie *et al.*, 1999)

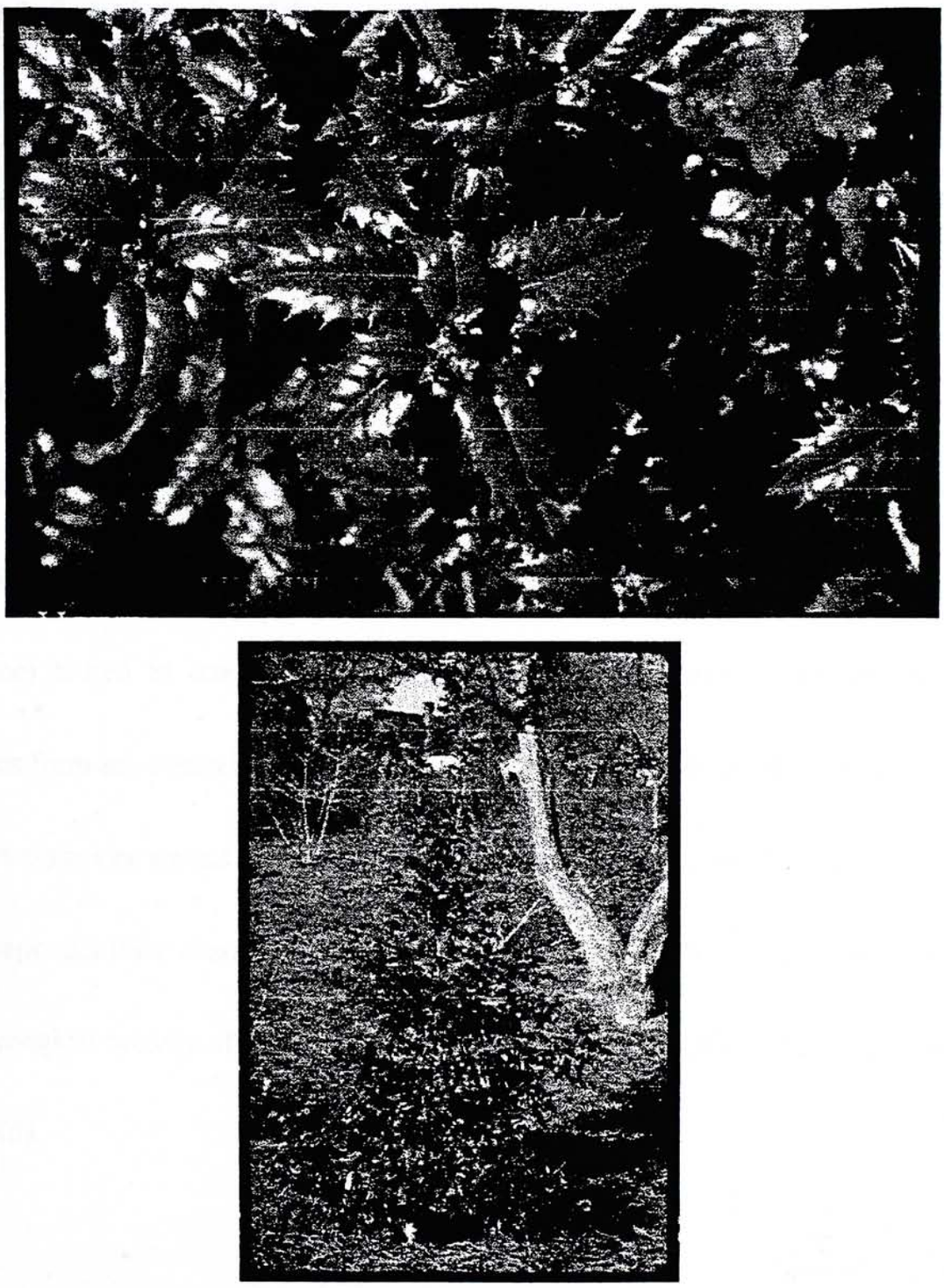

Figure 1.3 The upper photo showing IL leaves and lower photo showing an IL plant

(Maackenzie *et al.*, 1999)

in IL. From some preliminary studies, IL contains saponins, polyphenols, amino acids, vitamin C and other chemicals. The chemical composition of IL is shown in Table 1.3. Saponin is one of the major components in IL. The medicinal effect of IL may be related to the large amount of saponin present. Therefore saponin-enriched fraction (SEF) of IL is worthy of investigation.

1.8 Saponin

Saponins are a structurally diverse group of naturally occuring compounds found mainly in plants. Chemically they consist of a steroid or triterpene group (the aglycone) linked to one or more sugar molecules. The structure of one of the saponins from soya bean is shown in Figure 1.4. The molecules are thus amphiphilic, the triterperene or steroid part being hydrophobic and the sugar part hydrophilic. This gives saponins their characteristic surface activity from which the name is derived. The biological activity of saponin is closely related to its specific structure (Sidhu et al., 1986).

1.9 Saponins in food and its biological effects

Saponins have been identified in many hundreds of plant species but only a few of them are used as food by man. Some of them and their saponin content is shown

Table 1.3 Preliminary analysis of chemical composition in IL (Li *et al.*, 1996)

Chemical	Content
Saponin	21-22%
Polyphenol	7.76-9.01%
Water soluble carbohydrates	6.99%
Alkaloids	1%
Vitamin C	0.031%-0.045%
Amino acids	0.14%
Special chemicals found in IL: taraxerol, ursolic acid, α-amyrin, β-amyrin, β-sitosterpol, latifolosides A,B,C,D and E.	

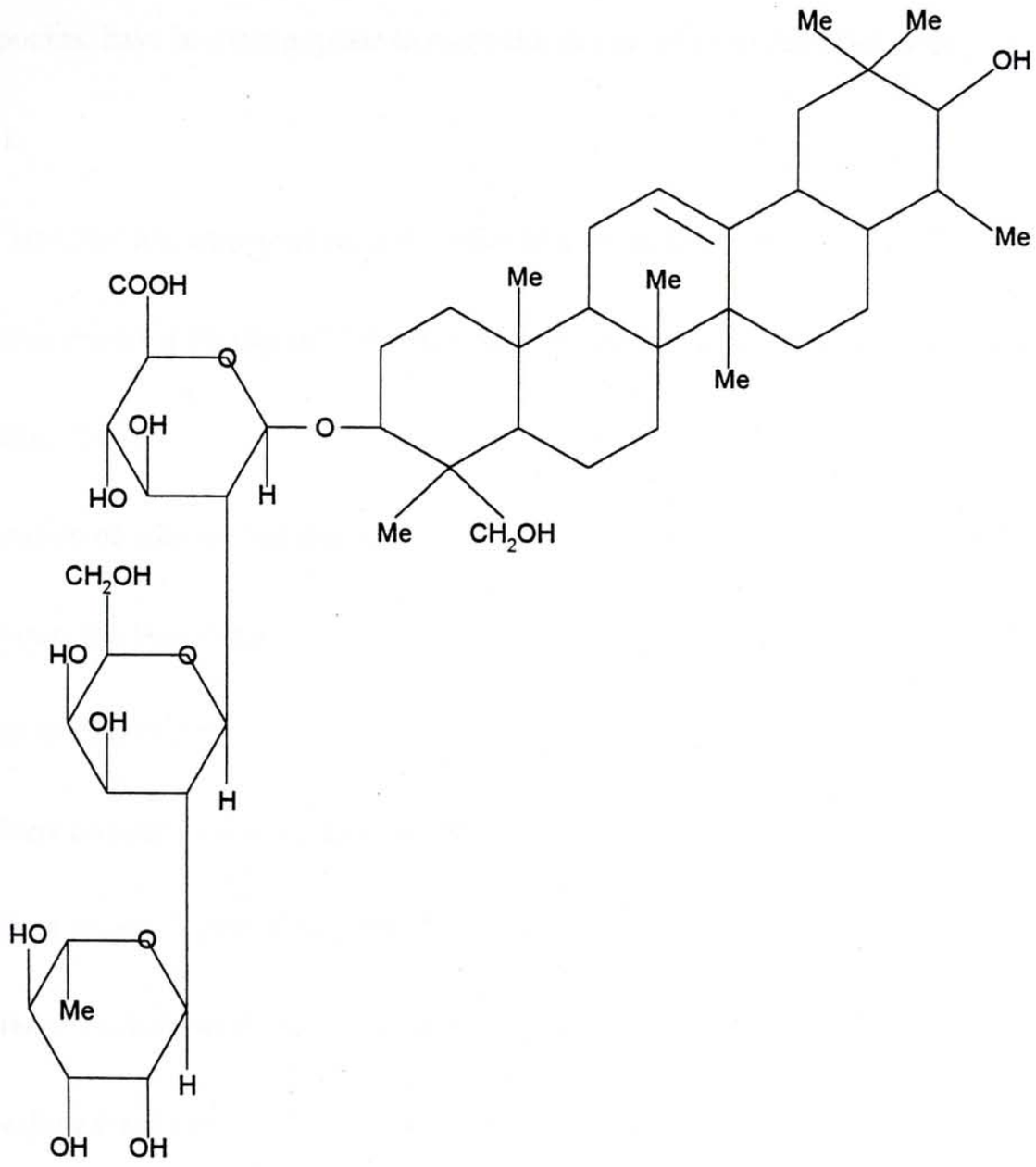

Figure 1.4 The structure of a saponin from soya bean (Sidhu *et al.*, 1986)

in Table 1.4. Most of the saponin-containing food plants are legumes including chickpeas, lentils, soy beans and peanuts. Alfalfa sprouts, which are particularly rich in saponins, have become popular in Australia as a salad vegetable (Oakenfull, 1990).

Saponins are known as natural surfactants. In addition to its physical property, saponins exhibit a variety of biological activities and some of them are listed below (Cheeke, 1983):

1. Irritation of mucous membranes
2. Erythrocyte hemolysis
3. Enzyme inhibition
4. Effects on feed intake and diet palatability
5. Effects on absorption of nutrients
6. Effects on cholesterol and bile acid metabolism
7. Antifungal activity
8. Anti-allergic effects

It indicates that saponins may have significant effects on all phases of animal production, from the ingestion of feed to the excretion of wastes. They also have a role in plants in protection against insects and disease. Alfalfa saponins protect the plant against numerous pathogenic fungi, particularly in the root (Cheeke, 1983).

Table 1.4 Plant foods and their saponin content (Oakenfull *et al.*, 1990)

Plant	Saponin content (g/kg)
Alfalfa sprouts (*Medicago sativa*)	80
Chickpea (*Cicer arietinum*)	2.3-60
Soya bean (*Glycine max*)	5.6-56
Navy bean (*Phaseolus vulagaris*)	4.5-21
Lentil (*Lens culinaris*)	1.1-5.1

1.10 The saponins in IL

As mentioned before, saponin is one of the major components in IL. Different triterpenoid saponins can be found in IL (Ouyang, *et al.*, 1997; Ouyang, *et al.*, 1998). Their structures are shown in Figure 1.5.

Saponins exhibit a variety of biological activities and have been investigated for development of new natural medicines and to prove the efficacy of traditional herbal medicine.

1.11 Outline of the thesis

IL is one of major parts of trilex tea and is a kind of kudingcha. IL has been stated to have some medicinal benefits. Scientific evidence to support the statement is lacking. Besides saponins exhibit some biological activities. It is rich in IL and may contribute to its medicinal benefits. Therefore it is worth studying.

In the present investigation, the toxicity and detoxification of IL and SEF were tested. The hypoglycemic and hypolipidemic effects of IL were also examined to explore its medicinal benefits.

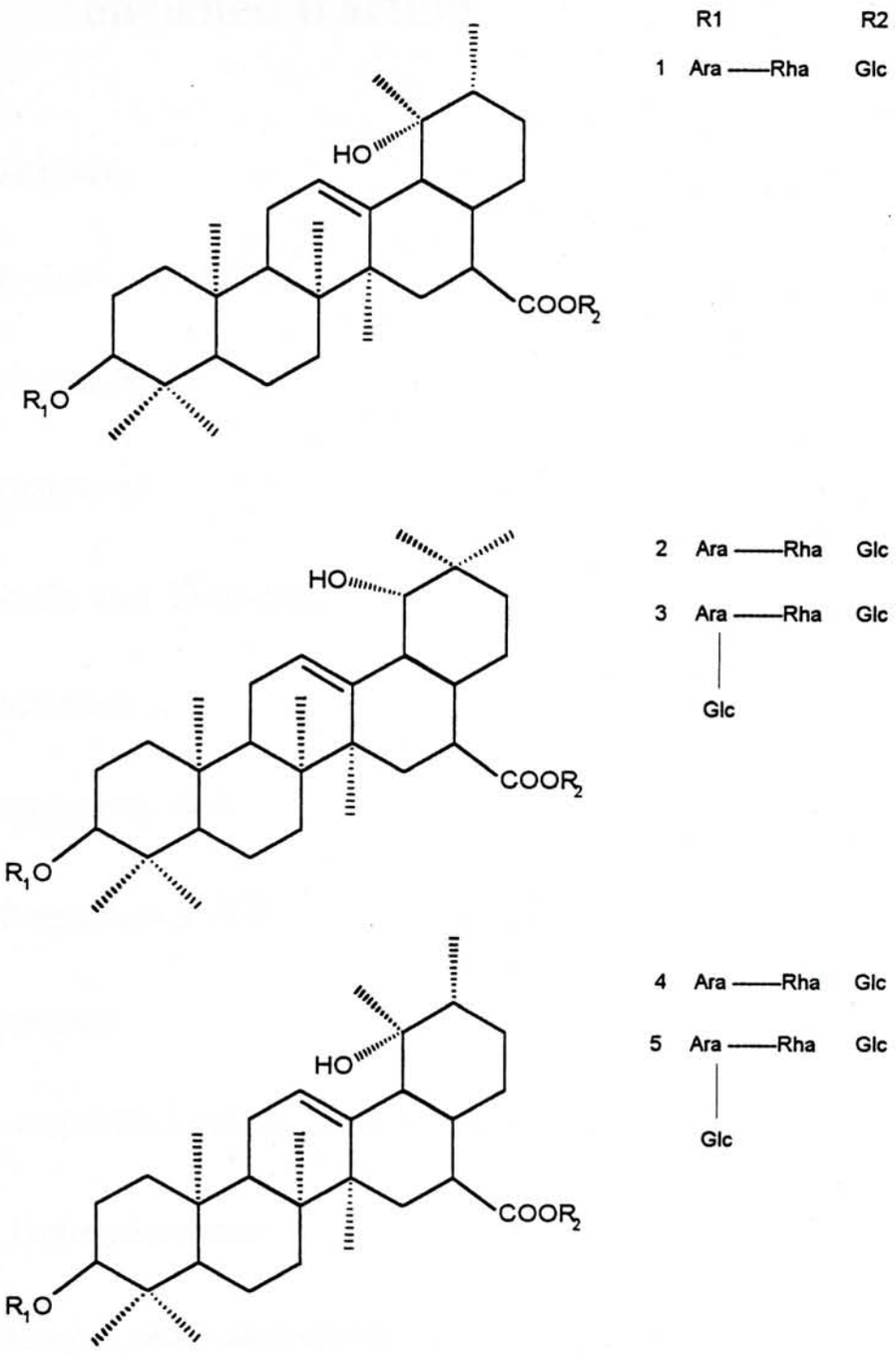

Figure 1.5 The structure of five triterpenoid saponins in IL.

Latifoloside A(1), B(2), C(3), D(4) and E(5) (Ouyang *et al.*, 1987)

Chapter 2 Toxicological studies on the effect of *Ilex latifolia* extract and its saponin-enriched fraction

2.1 Introduction

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2.3.6 Sub-chronic effect of IL and SEF on various xenobiotic enzymes in normal mice

2.4 Discussion

2.1 Introduction

2.1.1 Toxicity of herbal tea

Individuals who attempt to reduce their caffeine intake often consume herbal tea – "natural" beverages that enjoy a reputation as being particularly " health food "products (Hiscoe, 1983). Over the past decade, however, at least 26 herbal tea have been found to be responsible for gastrointestinal, hepatic, hematologic and nervous system abnormalities (Ridker, 1987). Five of them are shown in Table 2.1. Most of these tea are commercially available, although exposure to toxic herbal tea has also followed misidentification of plant material as that cultivated at home. Within the United States, four fatalities have been reported from herbal tea poisoning (Ridker, 1987). Nowadays the growing consumption of health food and exposure to potentially toxic herbal products can be expected to increase. Therefore toxicity tests of different kinds of herbal teas are of critical importance.

2.1.2 Hepatotoxicity

The liver plays a major role in the detoxification of xenbiotics for protection. Some herbal teas, mentioned in Table 2.1, were suspected to cause hepatic failure by interfering with the detoxification process. Therefore testing the toxicity of herbal teas on the liver is important. Aspartate aminotransferase (AST) and alanine

Table 2.1 Potential toxicity of herbal tea (Ridker, 1987)

Tea constituent	Botanical source	Suspected toxin	Clinical toxicity
Buckthorn	*Hiptothae rhamnoides*	anthraquinones	Cathartic toxin Severe watery
Comfrey	*Symphytum officinale*	Pyrrolizidine alkaloids	Hepatic failure
Hops	*Humulus lupulus*	lupuline	Intravascular hemolysis
Mate	*Ilex paraguayensis*	Pyrrolizidine alkaloids	Hepatic failure

aminotransferase (ALT) are two biomarkers for liver damage. Since AST and ALT are present at high concentrations in liver cells, damage to the liver cells will cause the serum AST and ALT levels to increase. Therefore, a rise in serum AST and a rise in serum ALT are two sensitive indicators for liver cell damage.

2.1.3 Objectives

The aim of this investigation was to study whether acute and sub-chronic treatment with IL and SEF produced any liver damage in normal mice. The effects of IL and SEF on various xenobiotic enzymes were also tested to study their effects on the liver detoxification process.

2.2 Materials and Methods

2.2.1 Materials

Sodium chloride, sodium sulphate, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, $NADP^+$, bovine serum albumin, hydrochloric acid, sodium hydroxide, magnesium sulfate, methoxyresorufin, ethoxyresorufin, 1,2-dichloro-4 – nitrobenzene, glycine, sucrose, potassium chloride, EDTA, potassium phosphate and oxidized glutathione were obtained from Sigma St. Louis, Missouri, U.S.A.. Methanol and ethanol were obtained from New England Nuclear Life Science, U.S.A.

2.2.2 Preparation of IL

The dried leaves of *Ilex latifolia* (IL) were blended to form a powder. The powder was then soaked in 100 volumes of water (w/v), boiled and allowed to simmer for two hours. The solution was filtered and centrifuged. The residue was sprayed dry and ready for use.

2.2.3 Preparation of SEF

0.5g IL was defatted with 1L petroleum ether by soaking overnight. Then IL was extracted with methanol (2L) at 80°C for two hours. The extract was dried by

the rotatory evaporator. The residue was re-dissolved in water and extracted with water-saturated n-butanol. Then the n-butanol fraction was washed with water three times and dried with a rotatory evaporator. The residue was dissolved in water and freeze-dried to obtain the SEF.

2.2.4 Animals

Eight to twelve week-old male Balb/c mice were obtained from the Laboratory Animal Services Centre (LASEC) in the Chinese University of Hong Kong. The animals had free access to food and water before the experiments.

2.2.5 Acute effect and sub-chronic effect of IL and SEF in normal mice

The mice received an oral administration of IL or SEF for 1 day. The mice were killed 24 hours later to collect blood for determination of AST and ALT activity. The weights of the liver, heart and kidney were also recorded. The liver was stored at –70 °C before use in the MROD, EROD and GST assays.

For investigating the sub-chronic effect of IL and SEF, the mice received an oral administration of IL or SEF daily for two weeks. The remaining procedure was the same as the experiment investigating the acute toxic effect of IL and SEF described above.

2.2.6 Tissue preparation

Animals were killed to remove the liver. The liver was homogenized in 4 volumes of 0.1M sucrose containing 0.05M potassium chloride, 0.03M EDTA and 0.04M potassium phosphate (pH 7.2) using a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 12,000g for 20 min at 4°C. The supernatant obtained was centrifuged again at 105,000g for 60 min twice. The supernatant was collected for the determination of cytosolic GST activity (Section 2.2.7). The pellet obtained was resuspended in the homogenizing buffer by gentle homogenization with a loose-fitting Teflon pestle. The preparations were stored at -70°C before use in the MROD, EROD and microsomal GST assays (Section 2.2.7).

2.2.7 Enzyme and protein assays

(A) Methoxyresorufin O-demethylase (MROD) and ethoxyresorufin O-deethylase (EROD) actitives

The assay was performed according of Pohl and Fouts (1980). Twenty-five μg of protein was incubated in 0.5ml of an assay medium containing 0.1mM magnesium sulfate, 1.6mg bovine serum albumin, 1.5μM methoxyresorufin or ethoxyresorufin and 50mM potassium phosphate, pH7.5. The reaction was initiated by the addition of 150μl of a NADPH-regenerating system (0.2 unit of glucose-6-phosphate

dehydrogenase, 0.28mM $NADP^+$ and 2.5mM glucose-6-phosphate). After incubation at 37°C for 15 min, the reaction was terminated by precipitating the protein with 1ml methanol. After centrifugation, the supernatant fluid was taken for fluorescene measurement with an excitation wavelength of 530nm and an emission wavelength of 590nm. The amount of resorufin produced was determined with reference to the standard curve.

(B) Glutathione-S-transferase (GST)

GST activity was measured spectrophotometrically by following the absorbance increase at 345nm at 25 °C. GST activity was determined in a reaction mixture consisting of 5mM glutathione and 0.1M potassium phosphate (pH 8.5) containing 1mM 1,2-dichloro-4-nitrobenzene as substrate. The GST activity was calculated based on an extinction coefficient of $8,500M^{-1}cm^{-1}$ for 1,2-dichloro-4-dinitrobenzene. A complete assay mixture without enzyme was used as a control.

(C) Alanine aminotransferase and aspartate aminotransferase

The blood collected in section 2.2.5 was coagulated for 45 min in room temperature and then centrifuged at 2500 rpm for 5 min at 4°C to obtain serum. Serum AST and ALT enzyme activities were determined by using commercial

enzyme kits from Sigma. ALT or AST reagents were prepared according to the instructions of Sigma kit. Serum (0.1ml) was added into AST or ALT reagent, mixed immediately and incubated at 30 °C for 90 seconds. Then the absorbance was read and recorded at 340nm as an initial absorbance. Water was used as reference. The incubation was continued for 60 seconds at 30°C. Absorbances at 30 seconds and 60 seconds were recorded at 340nm. The absorbance reading at 60 seconds was final absorbance. The change of absorbance per min, obtained by subtracting final absorbance and initial absorbance can be determined to calculate AST and ALT activities.

(D) Protein determination

Protein was determined by the method of Lowry *et al.*, (1951), using bovine serum albumin as the standard. 0.1 ml sample (buffer or standard) was mixed with 2 ml reagent A which contains 1 volume of 1% $CuSO_4*5H_2O$, 1 volume of 2% NaK-tartrate and 100 volumes of 2% Na_2CO_3 in 0.1M NaOH. The reaction mixture was allowed to stand for 10 min. 0.1ml Folin-Ciocalteu's phenol reagent was added and mixed immediately. The mixture was allowed to stand for 45 min before the absorbance at 750nm was measured.

2.2.8 Statistical analysis

Statistical analysis was performed by using the Student's t-test. A p value of 0.05 or below indicates statistically significant difference.

2.3 Results

2.3.1 Acute effect of IL and SEF as reflected in the body weight and weights of other tissues in normal mice

The results of IL and SEF treatment on the body weight and weights of other tissues are shown in Tables 2.3.1 and 2.3.2 respectively. The body weights of the mice were maintained in all groups receiving IL. However the liver weights of mice receiving IL (1g/kg) and IL (3g/kg) underwent a slight decrease. The differences were not statistically significant, however. Besides the weights of the heart and the kidney in all groups were also constant. The body, liver, heart and kidney weights of mice in the SEF group were also similar to those of the control.

2.3.2 *Sub-chronic effect of IL and SEF as reflected in body weight and weights of other tissues in normal mice*

The results on IL and SEF treatment on the body weight and weight of other tissues are shown in Tables 2.3.3 and 2.3.4 respectively. Compared with the control, the body weights of all IL treated group were slightly increased. The liver weights in all IL treated groups were higher than those of control. However all of them showed no statistically significant difference. There was no difference between all IL treated groups and control in the weight of the kidney and heart. The body, liver, kidney and

Table 2.3.1 Acute treatment with different concentrations of IL on body weight and liver, kidney and heart weights in mice

	Control	IL (0.3g/kg)	IL (1g/kg)	IL (3g/kg)
Body Weight (g)	20.2±0.7	20.4±0.8	20.3±1.0	20.2±1.3
Liver Weight (g)	0.99±0.03	0.99±0.07	0.95±0.04	0.95±0.09
Liver Weight (% of B.W.)	4.92±0.05	4.86±0.25	4.65±0.03	4.69±0.16
Kidney Weight (g)	0.31±0.02	0.30±0.02	0.29±0.02	0.30±0.02
Kidney Weight (% of B.W.)	1.53±0.03	1.47±0.06	1.42±0.03	1.47±0.02
Heart Weight (g)	0.10±0.00	0.09±0.01	0.10±0.01	0.10±0.01
Heart Weight (% of B.W.)	0.47±0.02	0.44±0.01	0.49±0.06	0.48±0.03

The mice received either water or IL at different concentration once. Their body weight, liver weight, kidney weight and heart weight were recorded at the end of the experiment which lasted for 1 day. All values are presented as means ± S.D. of 5 mice. No statistically significant change due to IL were detected.

Table 2.3.2 Acute effects of SEF on the body weight and liver, kidney and heart weights in mice

	Control	SEF (100mg/kg)
Body Weight (g)	20.7±0.7	20.9±0.8
Liver Weight (g)	0.99±0.07	0.98±0.03
Liver Weight (% of B.W.)	4.79±0.45	4.70±0.12
Kidney Weight (g)	0.29±0.01	0.30±0.01
Kidney Weight (% of B.W.)	1.41±0.07	1.45±0.09
Heart Weight (g)	0.10±0.00	0.10±0.01
Heart Weight (% of B.W.)	0.48±0.02	0.47±0.05

The mice received either water or SEF once. Their body weight, liver weight, kidney weight and heart weight were recorded at the end of the experimental period which lasted for 1 day. All values are presented as means ± S.D. of 5 mice. No statistically significant change due to SEF were detected.

Table 2.3.3 Sub-chronic effect of different concentrations of IL on body weight and liver, kidney and heart weights in mice

	Control	IL (0.3g/kg)	IL (1g/kg)	IL (3g/kg)
Body Weight (g)	19.4±0.8	19.7±1.2	20.1±0.7	19.8±0.7
Liver Weight (g)	1.02±0.05	0.96±0.03	1.01±0.06	0.97±0.05
Liver Weight (% of B.W.)	5.29±0.24	4.87±0.24	5.02±0.29	4.90±0.28
Kidney Weight (g)	0.30±0.01	0.30±0.01	0.29±0.01	0.30±0.01
Kidney Weight (% of B.W.)	1.55±0.06	1.51±0.10	1.46±0.01	1.50±0.05
Heart Weight (g)	0.10±0.00	0.10±0.01	0.10±0.01	0.10±0.01
Heart Weight (% of B.W.)	0.50±0.04	0.52±0.05	0.48±0.03	0.50±0.04

The mice received either water or IL at different concentrations once a day for 14 days. Their body weight, liver weight, kidney weight and heart weight were recorded at the end of the experimental period. All values are presented as means ± S.D. of 5 mice. No statistically significant change due to IL were detected.

Table 2.3.4 Sub-chronic effects of SEF on body weight and liver, kidney and heart weights in mice

	Control	SEF (100mg/kg)
Body Weight (g)	20.5±1.3	20.6±1.2
Liver Weight (g)	1.00±0.08	1.06±0.10
Liver Weight (% of B.W.)	4.91±0.50	5.16±0.63
Kidney Weight (g)	0.29±0.02	0.28±0.02
Kidney Weight (% of B.W.)	1.44±0.17	1.38±0.15
Heart Weight (g)	0.10±0.01	0.10±0.01
Heart Weight (% of B.W.)	0.51±0.05	0.47±0.02

The mice received either water or SEF once a day for 14 days. Their body weight, liver weight, kidney weight and heart weight were recorded at the end of the experimental period. All values are presented as means ± S.D. of 5 mice. No statistically significant change due to SEF were detected.

heart weights in SEF-treated were similar to those in control animals.

2.3.3 Acute effects of IL and SEF on the AST and ALT activities in normal mice

The results for acute effect of IL and SEF on AST and ALT activities are shown in Tables 2.3.5 and 2.3.6 respectively. The normal ranges of AST and ALT in Balb/c mice were 50-200 μmole/min/L and 30-150μmole/min/L respectively. The AST activities in all IL-treated groups were within 15 % compared with control. ALT in all IL-treated groups was similar to that of control. The AST and ALT levels in SEF treated group were also similar to those of the control. All AST and ALT level in two sets of experiments were within the normal range.

2.3.4 Sub-chronic effect of IL and SEF on AST and ALT activities in normal mice

The results for sub-chronic effect of IL and SEF on AST and ALT activities are shown in Table 2.3.7 and 2.3.8. After administration of IL for two weeks, AST level in IL (1g/kg) and IL (3g/kg) groups were slightly increased. However the AST level in all IL treated groups were within 20 % compared with the control and the difference were not statistically difference. ALT level in all IL treated groups were similar to that of control. AST and ALT levels in SEF treated group were also similar to those of control. All data were within the normal range.

Table 2.3.5 Acute effect of different concentrations of IL on serum AST and ALT activities in mice

	Serum enzyme activity (μmole/min/L)	
	AST	ALT
Control	104.1±31.9	46.8±9.2
IL (0.3g/kg)	95.7±22.7	44.9±11.7
IL (1g/kg)	93.7±22.6	37.1±5.9
IL (3g/kg)	93.9±22.0	36.7±2.6

The mice received either water or IL (different concentrations) once. Enzyme activities were determined as described in Materials and Methods. All values are presented as means ± S.D. of 5 mice. No statistically significant change due to IL were detected.

Table 2.3.6 Acute effect of SEF on serum AST and ALT activities in mice

	Serum enzyme activity (μmole/min/L)	
	AST	ALT
Control	89.2±10.1	43.3±6.8
SEF (100mg/kg)	85.2±18.2	49.3±5.3

The mice received either 5% ethanol or SEF once. Enzyme activities were determined as described in Materials and Methods. All values are presented as means ± S.D. of 5 mice. No statistically significant change due to SEF were detected.

Table 2.3.7 Sub-chronic effect of different concentrations of IL on serum AST and ALT activities in mice

	Serum enzyme activity (μmole/min/L)	
	AST	ALT
Control	86.7±18.6	44.4±8.7
IL (0.3g/kg)	88.1±23.1	43.5±5.0
IL (1g/kg)	103.4±15.8	46.5±6.0
IL (3g/kg)	94.4±24.5	42.5±9.8

The mice received either water or IL (different concentrations) once a day for 14 days. Enzyme activities were determined as described in Materials and Methods. All values are presented as means ± S.D. of 5 mice. No statistically significant change due to IL were detected.

Table 2.3.8 Sub-chronic effect of SEF on serum AST and ALT activities in mice

	Serum enzyme activity (μmole/min/L)	
	AST	ALT
Control	94.6±12.5	39.6±8.2
SEF (100mg/kg)	87.9±13.1	44.9±10.2

The mice received either 5% ethanol or SEF daily for 14 days. Enzyme activities were determined as described in Materials and Methods. All values are presented as means ± S.D. of 5 mice. No statistically significant change due to SEF were detected.

2.3.5 Acute effect of IL and SEF on various xenobiotic enzymes in normal mice

The results of IL and SEF are shown in Tables 2.3.9 and 2.3.10 respectively.

MROD: Compared with the control, all IL treated groups had similar MROD activity. The result of SEF-treated group on MROD activity was similar to that of IL. They had no induction effect on MROD activity.

EROD: Compared with the control, all IL treated groups had no induction effect in EROD activity. The result of SEF group was similar to that of IL treated groups.

GST: Compared with the control, both IL (1g/kg) and IL (3g/kg) significantly induced cytosolic GST activity by 47% and 52% respectively. They also significantly induced microsomal GST activity by 31 % and 43 % respectively. IL (0.3g/kg) also slightly but not significantly induced cytosolic and microsomal GST activity. SEF did not alter cytosolic and microsomal GST activities.

Table 2.3.9 Acute effect of different concentrations of IL on the activities of various detoxifying enzymes in mice

	Enzyme activity			
	MROD	EROD	GST (cytosolic fraction)	GST (microsomal fraction)
	pmole/min/mg	pmole/min/mg	μmole/min/mg	μmole/min/mg
Control	358±40	275±41	2.28±0.38	5.00±0.55
IL(0.3g/kg)	387±95	339±57	2.79±0.56	5.38±1.29
IL(1g/kg)	317±61	301±47	3.37±0.63*	6.56±0.86**
IL(3g/kg)	403±56	276±32	3.47±0.81**	7.15±1.22**

Mice received either water or IL at different concentrations for 1 day. Enzyme activities were determined as described in Materials and Methods. All values are presented as means ± S.D. of 5 mice.

*$p < 0.05$, **$p < 0.01$, significantly different from control by Student's t-test.

Table 2.3.10 Acute effect of SEF on the activities of various detoxifying enzymes in mice

	Enzyme activity			
	MROD	EROD	GST (cytosolic fraction)	GST (microsomal fraction)
	pmole/min/mg	pmole/min/mg	μmole/min/mg	μmole/min/mg
Control	379±67	398±58	2.22±0.29	4.48±0.66
SEF (100mg/kg)	435±53	410±34	2.41±0.27	4.79±0.58

Mice received either water or SEF for 1 day. Enzyme activities were determined as described in Materials and Methods. All values are presented as means ± S.D. of 5 mice. No statistically significant change due to SEF were detected.

2.3.6 Sub-chronic effects of IL and SEF on various xenobiotic enzymes in normal mice

The sub-chronic effects of IL and SEF are shown in Tables 2.3.11 and 2.3.12.

MROD: After treatment with IL at different concentrations for two weeks, only IL at the dose of (1g/kg) elevated MROD activity by 15 %. The increase was not statistically significant. Compared with the control, SEF had no induction effect on MROD.

EROD: Compared with the control, different doses of IL had no augmenting effect on EROD. SEF also yielded similar results.

GST: All dosages of IL significantly induced cytosolic GST activity. IL (1g/kg) and IL (3g/kg) induced cytosolic GST activity by about 77% and 72% respectively. IL (0.3g/kg) induced cytosolic GST activity by 43%. For the microsomal GST activity, IL (1g/kg) and IL (3g/kg) significantly induced GST activity by 46% and 54% respectively. IL (0.3g/kg) induced GST activity by 23% although the change was not statistically significant. SEF had no induction effect on cytosolic and microsomal GST activity.

Table 2.3.11 Sub-chronic effects of different concentrations IL of on the activities of various detoxifying enzymes in mice

	Enzyme activity			
	MROD	EROD	GST (cytosolic fraction)	GST (microsomal fraction)
	pmole/min/mg	pmole/min/mg	μmole/min/mg	μmole/min/mg
Control	363±58	310±44	2.17±0.64	4.86±0.75
IL(0.3g/kg)	385±59	319±39	3.11±0.74*	6.04±1.43
IL(1g/kg)	420±76	325±26	3.85±0.76**	7.12±1.03***
IL(3g/kg)	383±52	339±40	3.74±061***	7.50±1.07***

Mice received either water or IL at different concentrations for 14 days. Enzyme activities were determined as described in Materials and Methods. All values are presented as means ± S.D. of 5 mice.

*p< 0.05, **p<0.01, ***p<0.005, significantly different from control by Student's t-test.

Table 2.3.12 Sub-chronic effect of SEF on the activities of various detoxifying enzymes in mice

	Enzyme activity			
	MROD	EROD	GST (cytosolic fraction)	GST (microsomal fraction)
	Pmole/min/mg	pmole/min/mg	μmole/min/mg	μmole/min/mg
Control	279±22	292±32	2.14±0.26	4.75±0.50
SEF(100mg/kg)	308±36	276±30	2.22±0.18	4.34±0.48

Mice received either water or SEF for 14 days. Enzyme activities were determined as described in Materials and Methods. All values are presented as mean ± S.D. of 5 mice. No statistically significant change due to SEF were detected.

2.4. Discussion

Weight loss is one indicator for toxicity. One example is that caused by administration of a high dose of simvastatins for twelve days, which can be used to treat hypercholesterolemia, caused liver necrosis with centrolobular degeneration, along with anorexia, weight loss and death (Diaz-Zagoya *et al.*, 1999). In the present study, oral administration of IL and SEF for one day, there was no significant change in body weight and liver weight. The results of IL and SEF after two weeks were the same as that of IL and SEF after one day. Besides, administration of IL and SEF also had no effect on the heart and kidney weights. It indicated that administration of IL and SEF had no lethal effect.

In the present investigation, oral administration of IL for one day and two weeks had no effect on serum levels of AST and ALT. According to Kong (unpublished data), oral administration of IL for 45 days and 90 days also had no effect on serum AST and ALT activities. The results indicated that acute, sub-chronic and chronic administration of IL did not cause any liver cell damage. Besides IL did not exert any effect on the body weight and weight of other tissues. It indicated that chronic administration of IL did not cause any observable damage in important tissues.

Saponins have a reputation for being highly toxic. It is because saponins possess the ability to haemolyze red blood cells (Butler *et al.*, 1973). They tend to alter the permeability of the cell wall and therefore exert a general toxicity on many organized

tissues. However the toxicity of saponin administered orally is low except for the saponin from common corn cockle (*Agostemma githage*). The oral lethal dose of the saponin from common corn cockle for dogs was 25-30mg/kg. The low toxicity of saponin is due to complete failure for it to cross the gut wall and enter the blood stream (Oakenfull *et al.*, 1990). Hence the hemolytic activity of saponin is reduced. It can also be reflected from the results regarding SEF (Sections 2.3.1 and 2.3.2). Oral administration of SEF did not cause any death after one day and after two weeks. SEF also had no effect on serum AST and ALT activities. It indicated that SEF did not cause any cell damage.

Xenobiotic chemicals may be considered to be non-nutritive, generally lipophilic, compounds that interact with organism to display toxic and sometimes carcinogenic effects. The duration and intensity of action of many xenobiotics within the biological systems are determined by the rate of their biotransformation to pharmacologically active or inactive products. The liver is the major organ for detoxifying xenobiotic chemicals to protect the body from damage. The detoxification of xenobiotic chemicals involves two phases: cytochrome P450 (phase I enzyme) and phase II enzyme. Cytochrome P450 is the major enzyme system responsible for the metabolism of many drugs and foreign chemicals to polar metabolites, thereby facilitating their elimination from the body (Wackett *et al.*, 1982;

Obermeier *et al.*, 1995). The existence of multiple forms of cytochrome P-450 is well known, and the composition of these isoforms in tissues as well as relative concentrations is influenced by treatment with different chemicals. The second step involves phase II enzymes such as GST, which can convert the metabolites into an soluble form for elimination. GST plays a physiological role in initiating the detoxification of potential alkylating agents, including pharmacologically active compounds (Habig *et al.*, 1974). These enzymes catalyze the reaction of compounds with –SH group of glutathione, thereby neutralizing their electrophilic sites and rendering the products more water-soluble. The glutathione conjugates can be further metabolized for excretion.

According to Zhong Yao Da Ci Dian (1977), one effect of IL is detoxicification. It may involve induction of certain liver enzymes. Therefore various xenobiotic enzymes were tested. After oral administration of IL for one day, none of the doses of IL had any effect on EROD or MROD activity. However IL (1g/kg and 3g/kg) induced both cytosolic and microsomal GST activity. The results of IL administration for two weeks were similar to those of IL administration for one day. They indicated that IL induced GST activity. As mentioned before, GST is one of the detoxifying enzymes. Induction of GST can enhance the removal of the metabolites of xenobiotics. Therefore the detoxifying effect of IL may be related to induction of

phase II enzymes.

SEF had no effect on the activities of MROD, EROD and GST. It may be related to the low solubility of SEF (Oakenfull *et al.*, 1989). Little SEF can enter into the blood stream and as a result it is difficult for SEF to affect the liver enzymes. Comparing the results of IL and SEF from two sets of experiment, administration for one day and administration for two weeks, it was found that only IL could induce the GST activity. Thus the effect of IL on enzyme induction may not be related to the saponin fraction. Some studies stated that polyphenols in green tea could induce GST activity (Bu-abbas *et al.*, 1998). Green tea is rich in polyphenol. IL also contains about 8 % of polyphenols, the effect of IL in inducing GST may be related to the presence of polyphenols.

In the present investigation, although SEF had no effect on the various xenobiotic enzymes, SEF may also contribute to a protective effect in other approaches. One example is the effect of α-hederin, which is a triterpenoid saponin found in herbs such as *Lonicera fulvotomentosa*. Cadmium (Cd) is an environmental pollutant which can cause liver damage. Liu *et al.* (1993) found that α-hederin induced metallothionein (MT) which is a metal binding protein for protection against the hepatotoxicity of Cd. It induced MT, which binds Cd in the cytosol thus reducing the amount of Cd in the critical cellular organelles and then decreasing the

hepatotoxicity by Cd. SEF can also be used to study the protection against the hepatotoxicants.

Chapter 3 Hypoglycemic effect of *Ilex latifolia* extract and its saponin-enriched fraction

3.1 Introduction

3.1.1 Diabetes

3.1.2 Herbal medicines

3.1.3 Objectives

3.2 Material and Methods

3.2.1 Materials

3.2.2 Animals

3.2.3 Induction of diabetes

3.2.4 Determination of serum glucose level

3.2.5 Effects on blood glucose level when IL or SEF was fed simultaneously with glucose

3.2.6 Effects on blood glucose level when IL or SEF fed 30 min before oral glucose load

3.2.7 Effects of IL or SEF on the serum glucose level in streptozotocin-induced diabetic mice

3.2.8 Effects of repeated administration of IL or SEF on serum glucose level in streptozotocin-induced diabetic mice

3.3 Results

3.3.1 Oral glucose tolerance test

3.3.2 Effect on serum glucose level when IL or SEF was fed 30 min prior to oral glucose load

3.3.3 Effect of IL or SEF on serum glucose level in streptozotocin-induced diabetic mice

3.3.4 Repeated administrations of IL or SEF on serum glucose level in streptozotocin-induced diabetic mice

3.4 Discussion

3.1 Introduction

3.1.1 Diabetes

Diabetes mellitus is a syndrome of disordered metabolism with inappropriate hyperglycemia due either to an absolute deficiency of insulin secretion or a reduction in the biological effectiveness of insulin. Diabetes mellitus can be classified into two major types according to the dependence on exogenous insulin. The two types of diabetes include (1) diabetes associated with insulin deficiency (Type I, insulin-dependent, IDDM) and (2) diabetes associated with insulin resistance (Type II, noninsulin-dependent, NIDDM) (Karan *et al.*, 1994).

The approaches to the control and prevention of hyperglycemia are central to the management of diabetes mellitus (Gray *et al.*, 2000). The development of new dietary adjuncts and novel antidiabetic agents, which reinstate a normal metabolic environment, thereby reducing the long-term complications associated with diabetes is required.

3.1.2 Herbal medicines

Throughout the world, many traditional plant treatments for diabetes exist and therein lies a hidden wealth of potentially useful natural products for diabetes control (Gray *et al.*, 1997a).

Recent studies showed that aqueous extracts of agrimony (*Agrimony eupatoria*), lucerne (*Medicago sativa*), coriander (*Coriandrum sativum*), eucalptus (*Eucalyptus globulus*) and edible mushroom (*Agaricus campestis*) enhanced insulin secretion and mimicked the effect of insulin on glucose metabolism *in vitro* (Gray *et al.*, 1997b; 1998a,b,c; 1999a,b). The dual pancreatic and extrapancreatic actions would prove to be an important advance on existing therapies used to treat and control diabetes, such as oral hypoglycemic drugs, which act either by enhancing insulin secretion, by improving the action of insulin or by interfering with glucose absorption and utilization. These combined findings illustrate the enormous potential of plants for use as possible dietary adjuncts and the discovery of natural products for diabetic therapy.

3.1.3 Objectives

The aim of this investigation was to study the acute hypoglycemic effect of IL and SEF on normal mice when fed with glucose simultaneously and when fed before glucose administration. The acute hypoglycemic effects of IL and SEF in diabetic mice were studied. Moreover the hypoglycemic effects of repeated administrations of IL and SEF on diabetic mice were also studied.

3.2 Materials and Methods

3.2.1 Materials

Streptozotocin, citric acid, ethanol, glucose and sodium citrate were obtained from Sigma. IL powder and SEF powder were obtained from the Biochemistry Department of the Chinese University of Hong Kong. Tolbutamide was obtained from Ko Wah apothecary.

3.2.2 Animals

Eight to twelve week-old male Balb/c mice were obtained from the Laboratory Animals Services Center in the Chinese University of Hong Kong. The animals had free access to food and water before the experiment.

3.2.3 Induction of diabetes

Diabetes was induced by an intraperitoneal streptozotocin injection after overnight fasting. Streptozotocin was dissolved in 0.5ml of freshly prepared sodium citrate buffer (10mM, pH4.5) and injected at a concentration of 100mg/kg body weight. Eight days after injection of streptozotocin, the serum glucose level of all the mice were determined. Mice with a serum glucose level above 250 mg/dl were considered to be diabetic and were used in the following experiments.

3.2.4. Determination of serum glucose level

Serum glucose level was determined by using an enzymatic kit from Sigma Chemical Co. The serum was diluted 20 fold with distilled water. Then 25 μl sample was added into 0.5 ml water and mixed with the Combined Enzyme-Color Reagent Solution from Sigma. Water and glucose standard solution were used as blank and standard respectively. The reaction mixture was incubated at 37°C for 30 min and then the absorbance was read at 450nm against the blank. The glucose concentration can be determined from the standard curve.

3.2.5. Effects on blood glucose level when IL or SEF was fed simultaneously with glucose

IL (0.3g/kg, 1g/kg and 3g/kg) or SEF (33mg/kg and 100mg/kg) with glucose 1.5g/kg were fed at 0 min to male mice which had been fasted overnight. Control mice were fed either water or 5 % ethanol with glucose. Mice fed with tolbutamide and glucose served as a positive control. The mice were killed at 0, 30, 60, 90, 120 min to determine the serum glucose level.

3.2.6 Effects on serum glucose level when IL or SEF was fed 30 min before the oral glucose load

IL (0.3g/kg, 1g/kg and 3g/kg) or SEF (33mg/kg and 100mg/kg) was fed 30 min before the experiment to mice which had been fasted overnight. The oral glucose load was given at time 0. Mice given either water or 5 % ethanol at 30 min before the experiment and then the glucose load was given at 0 min served as control. The group treated with tolbutamide was used as a positive control. Blood was collected at –30, 0, 30, 60, 90 and 120 min for determination of serum glucose level.

3.2.7. Effects of IL or SEF on the serum glucose level in streptozotocin-induced diabetic mice

The mice mentioned above in 3.2.3 were used. IL (0.3g/kg, 1g/kg and 3g/kg) or SEF (33mg/kg and 100mg/kg) was fed at 0 min to mice which had been overnight fasted. Blood was taken at different time points as in 3.2.6.

3.2.8. Effects of repeated administrations of IL or SEF on serum glucose level in streptozotocin-induced diabetic mice

Thirty-two mice (twenty-four diabetic mice and eight normal mice) were used in this study. They were housed, 8 mice per cage, in an animal room at constant

temperature (20-25°C) and with 12 hour light/dark cycles. The diabetic mice were fed water, IL (3g/kg) or SEF (100mg/kg) and tolbutamide (50mg/kg) daily for four weeks. The normal mice were treated with water as normal control. 5 % ethanol was used to substitute water for investigating the effect of SEF. Blood was collected after 4 weeks following overnight fasting. The body weights of the mice and the number of deaths of the mice were also recorded. Mice were given free access to tap water and food throughout entire experimental period.

3.3 Results

3.3.1 Oral glucose tolerance test

The serum glucose levels after IL treatment are shown in Table 3.3.1. The peak of serum glucose level appeared 30 min after oral administration of glucose. The glucose level returned to basal level after 120 min. The serum glucose level after treatment of IL (3g/kg) at 30 min was decreased by 10 % when compared with the normal control group and the difference was statistically significant ($p<0.05$). Serum glucose levels at 60 min and 90 min were also significantly reduced. Although IL (1g/kg) slightly decreased the serum glucose level at 30 min, the effect was not significant. IL at the dose of 0.3g/kg was not capable of lowering serum glucose level, indicating that only the highest concentration of IL reduced the serum glucose level in normal mice. Tolbutamide, which reduces serum glucose level by inducing insulin secretion, served as a positive control. As shown in Figure 3.3.1, its hypoglycemic effect was significant at all time points except 0 min.

The effects of SEF on oral glucose tolerance in normal mice are shown in Figure 3.3.2. Only saponin (100mg/kg) lowered serum glucose level significantly at all time points except time 0. The reduction in serum glucose level was about 10 % when compared with the normal control. Although SEF slightly reduced the serum glucose level at 30 min, the effect was not significant.

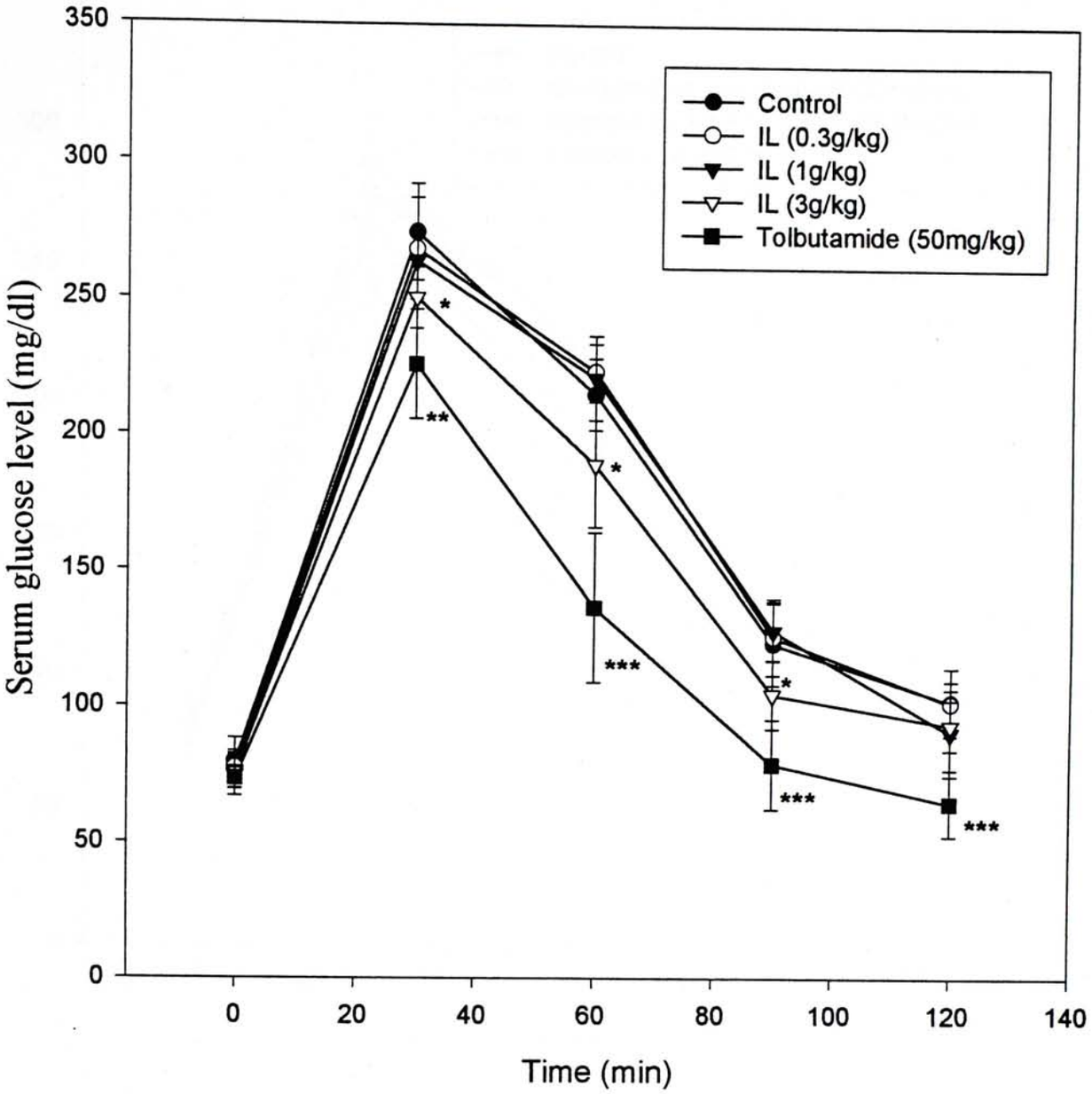

Figure 3.3.1 Effect of oral administration of different concentrations of IL on oral glucose tolerance in normal mice. Water, IL or tolbutamide was administered simultaneously with 1.5 g/kg glucose at time 0 after overnight fasting.

The serum glucose level was determined as described in Materials and Methods.* $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, significantly different from the corresponding control level. The data are presented as means $\pm$ S.D. N = 6 mice in each group.

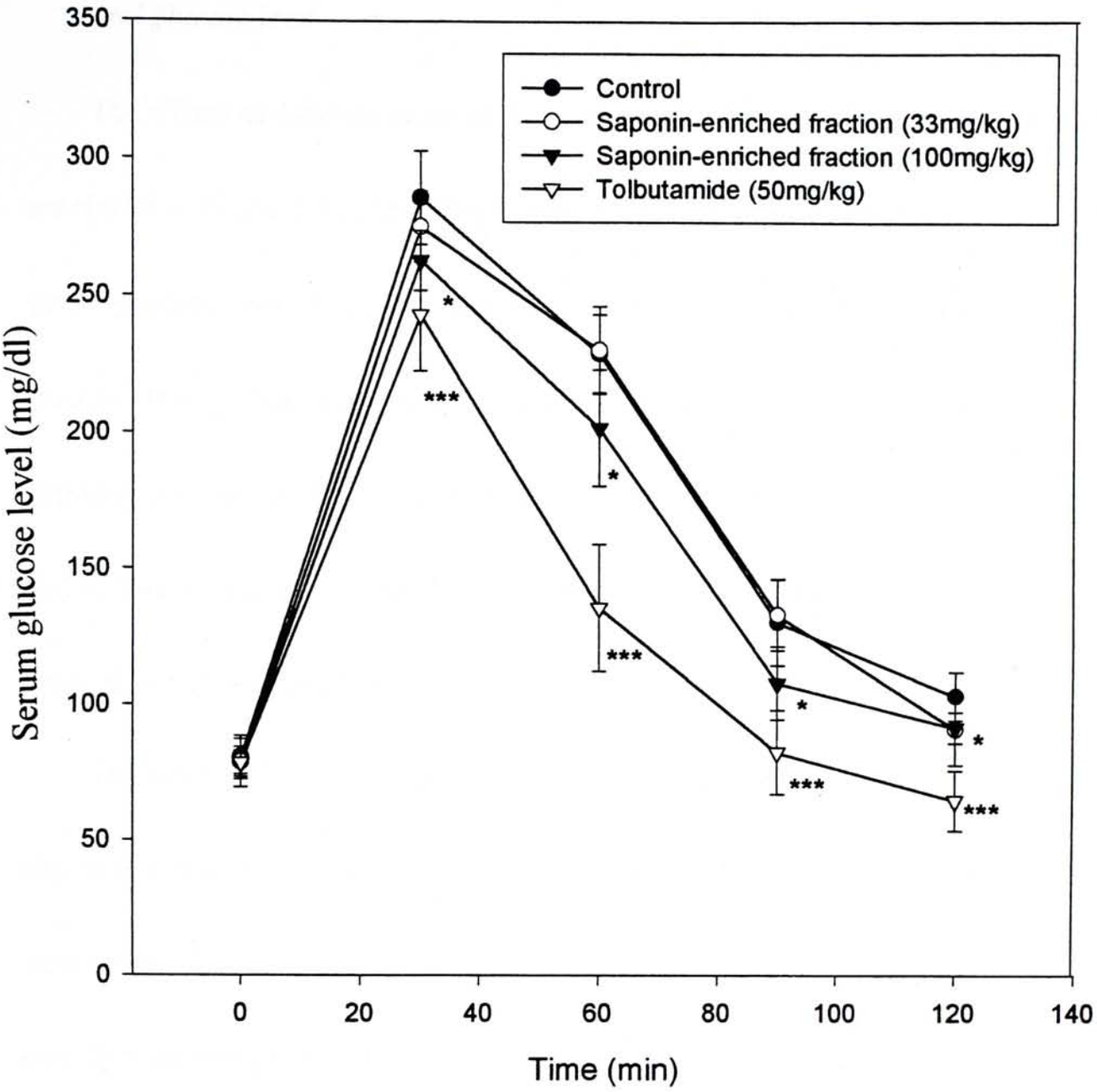

Figure 3.3.2 Effect of oral administration of different concentrations of SEF on oral glucose tolerance in normal mice. 5 % ethanol, SEF or tolbutamide was administered simultaneously with 1.5 g/kg glucose at time 0 after overnight fasting.

The serum glucose level was determined as described in Materials and Methods.* $p < 0.05$, *** $p < 0.005$, significantly different from the corresponding control level. The data are presented as means $\pm$ S.D. N = 6 mice in each group.

3.3.2 Effects on serum glucose level when IL or SEF was fed 30 min prior to oral glucose load

The effects of different doses of IL on serum blood glucose level in normal mice are shown in Figure 3.3.3. Only the highest dose of IL (3g/kg) significantly lowered serum glucose level at 30 min and 60 min. It brought about 14 % reduction in glucose level when compared with the normal control. It seems that the hypoglycemic effect of IL fed before the oral glucose load was stronger than that of IL fed simultaneously with glucose. However, the other dosages of IL only weakly reduced serum glucose level.

The effects of SEF on serum glucose level were also studied. The results are shown in Figure 3.3.4. The hypoglycemic effect of SEF was observed at the highest dose (100mg/kg) and the effect was significant at all time points except 0 min and 30 min. It could bring a reduction of about 14 % in serum glucose level. Compared with the effect of SEF shown in Figure 3.3.2, it exerted a stronger hypoglycemic effect. SEF at the dose of 33mg/kg also had a glucose lowering effect which was significant at 30 min.

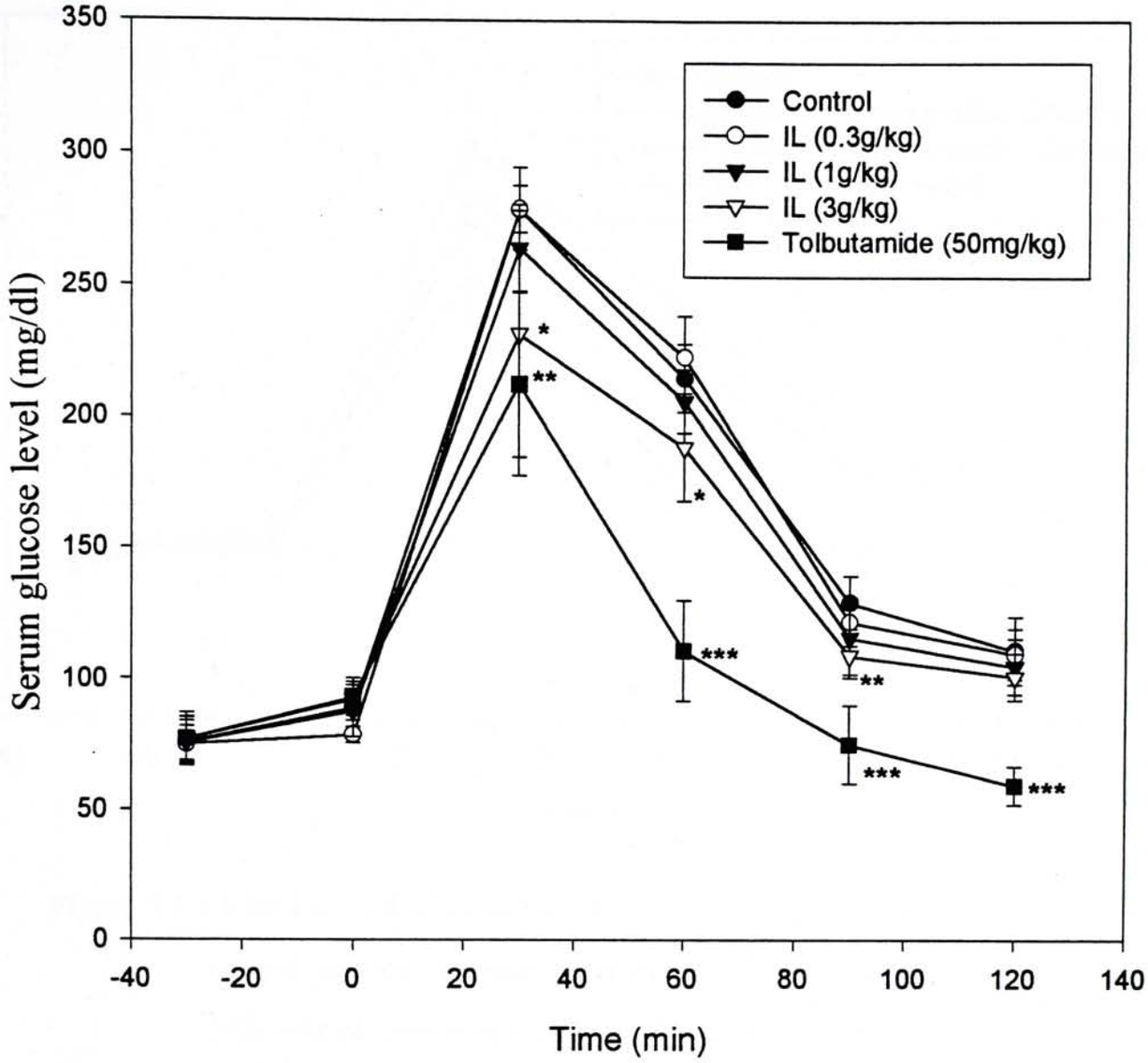

Figure 3.3.3 Effect of oral administration of different concentrations of IL on the oral glucose tolerance in normal mice. Distilled water or IL was administered orally at -30 min after overnight fasting and an oral load glucose (1.5g/kg) was given at time 0. Tolbutamide was also administered at time 0.

The serum glucose level was determined as described in Materials and Methods.* $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, significantly different from the corresponding control level. The data are presented as means $\pm$ S.D.. N = 6 mice in each group.

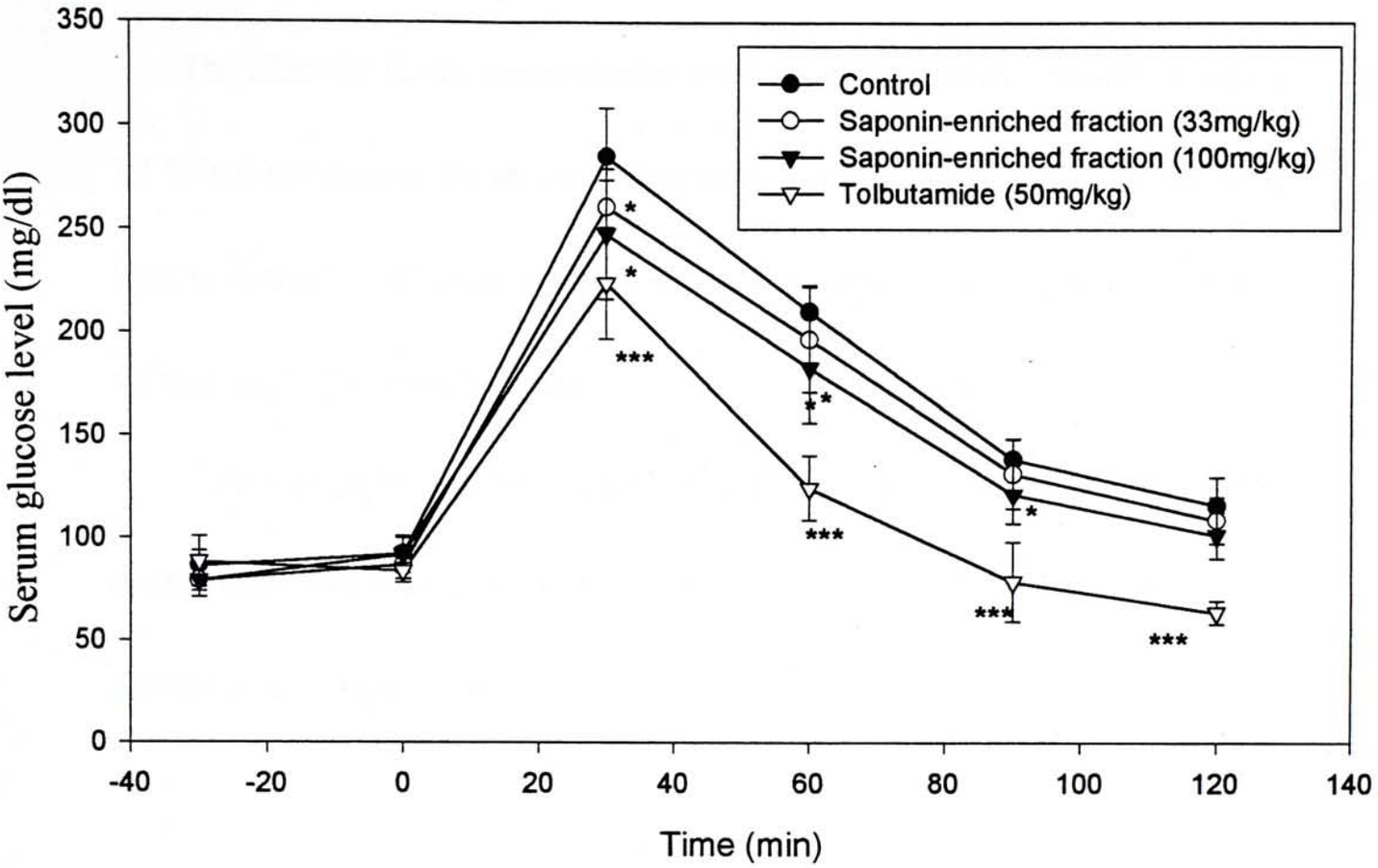

Figure 3.3.4 Effect of oral administration of different concentrations of SEF on oral glucose tolerance in normal mice. 5 % ethanol or SEF was administered orally at -30 min after overnight fasting and an oral load of glucose (1.5g/kg) was given at time 0. Tolbutamide was also administered at time 0.

The serum glucose level was determined as described in Materials and Methods. * $p < 0.05$, *** $p < 0.005$, significantly different from the corresponding control. The data are presented as means $\pm$ S.D.. N = 6 mice in each group.

3.3.3 Effect of IL or SEF on serum glucose level in streptozotocin-induced diabetic mice

The effect of IL on serum glucose level in diabetic mice is shown in Figure 3.3.5. In diabetic mice, the serum glucose level was three times higher than the basal level in normal mice. None of the doses of IL showed any hypoglycemic effect in diabetic mice. Tolbutamide (50mg/kg) reduced the serum glucose level.

The hypoglycemic effect of SEF in diabetic mice is shown in Figure 3.3.6. The results were similar to those of IL in Figure 3.3.5. SEF did not reduce the serum glucose level in diabetic mice.

3.3.4 Repeated administrations of IL or SEF on serum glucose level in diabetic mice

The effect of IL on serum glucose level is shown in Figure 3.3.7. IL at the dose of 3g/kg was orally administered to diabetic mice once daily for 4 weeks, and the fasting blood glucose level was determined after 4 weeks. Repeated administrations of IL produced a significant decrease in the fasting blood glucose level compared to the diabetic control at week 4, while the control group showed an increase in the glucose level from the pre-treatment level (week 0). IL brought about 20 % reduction in glucose level in diabetic mice. In the tolbutamide-treated group, the serum glucose

level showed a significant decrease when compared with the diabetic control group. IL also minimized the body weight change and the mortality when compared with the diabetic control. The results are shown in Table 3.3.1.

The effect of IL on serum glucose level in diabetic mice is shown in Figure 3.3.8. Repeated administrations of SEF brought about a significant decrease of about 17% in serum glucose level when compared with the diabetic control. SEF also minimized body weight decrease and mortality when compared with the diabetic control. The result is shown in Table 3.3.2.

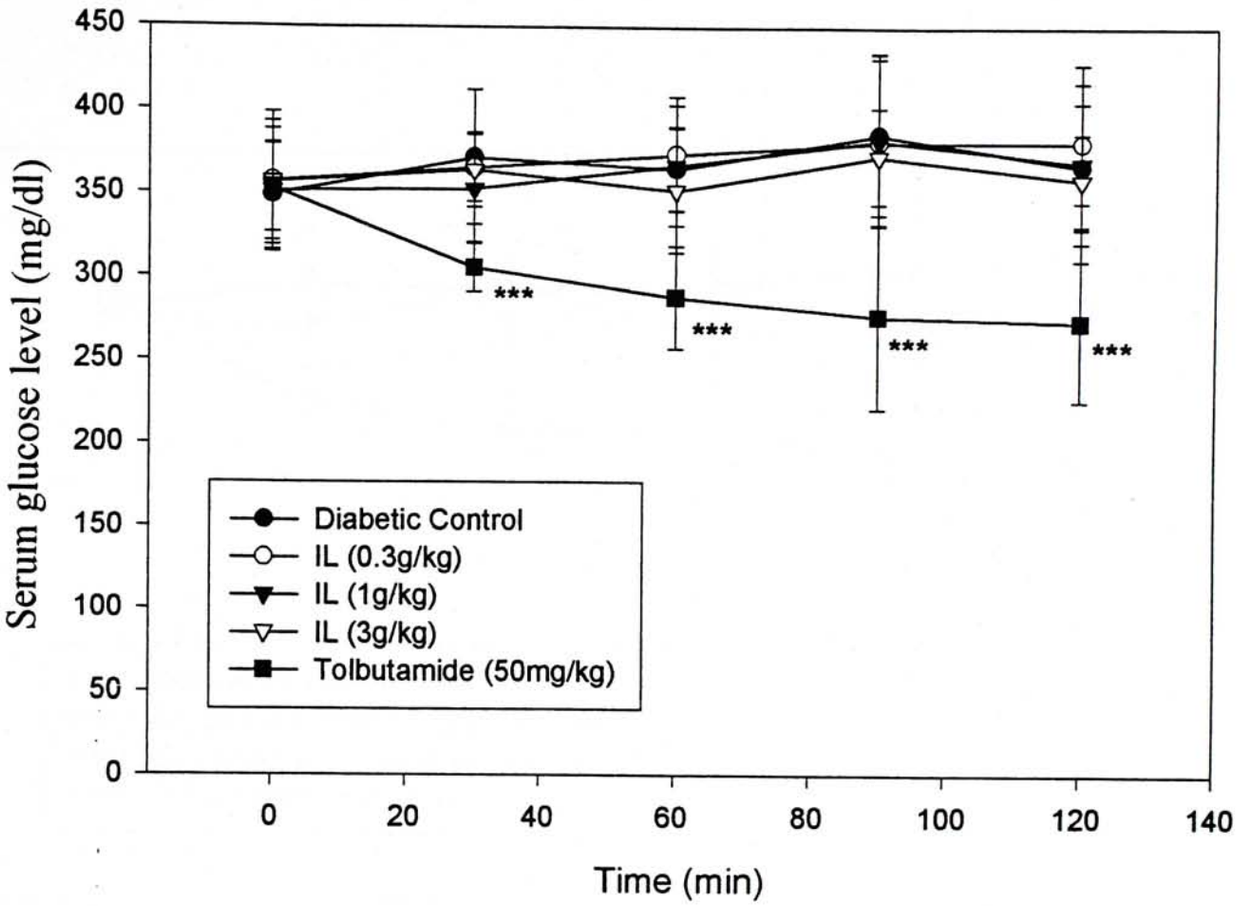

Figure 3.3.5 Effect of oral administration of different concentrations of IL on serum glucose level in streptozotocin-induced diabetic mice. Water, IL or tolbutamide was administered at time 0 after overnight fasting.

The serum glucose level was determined as described in Materials and Methods. *** $p < 0.005$, significantly different from the diabetic control. The data are presented as means $\pm$ S.D.

N = 6 mice in each group.

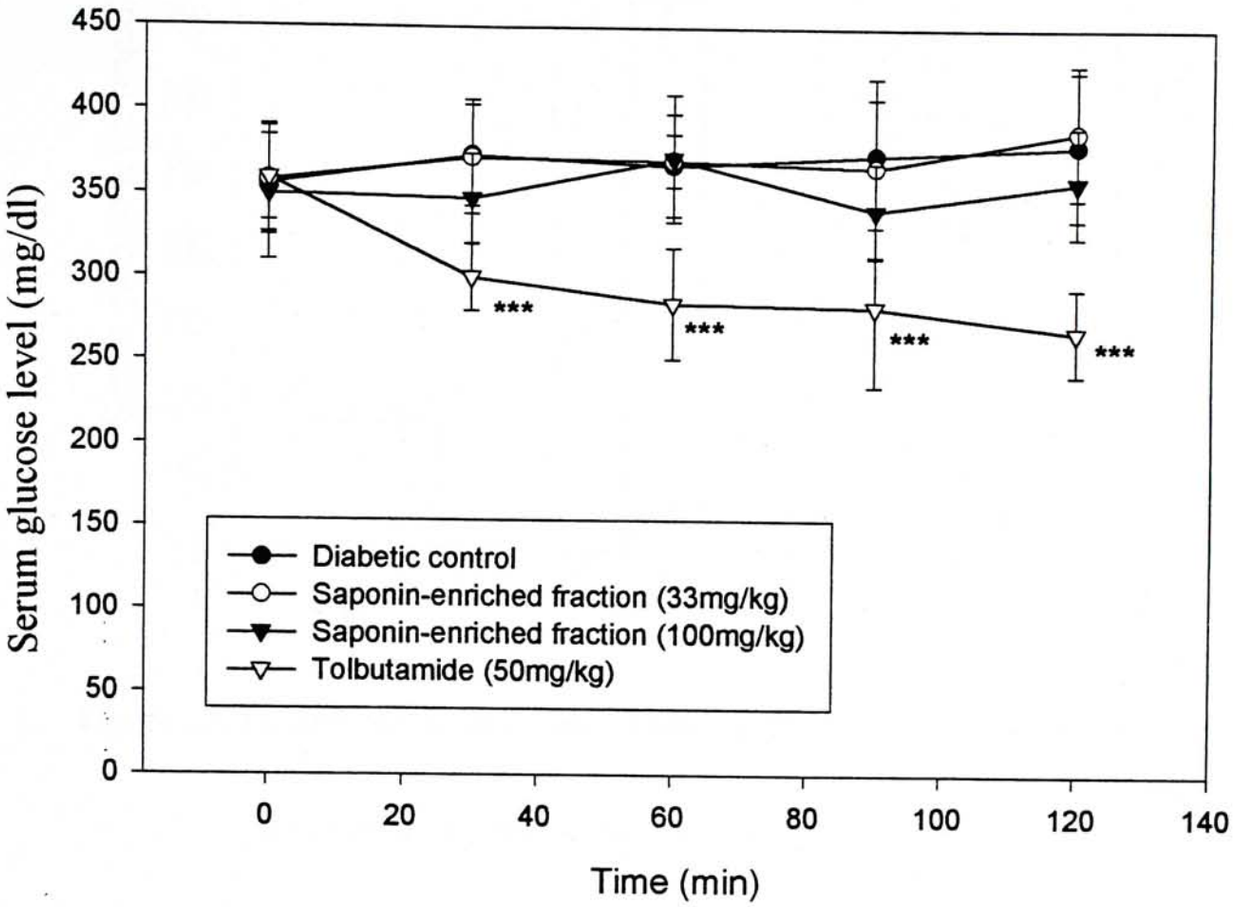

Figure 3.3.6 Effect of oral administration of different concentrations of SEF on glucose level in streptozotocin-induced diabetic mice. 5 % ethanol, SEF or tolbutamide was administered at time 0 after overnight fasting. The serum glucose level was determined as described in Materials and Methods. *** $p < 0.005$, significantly different from the diabetic control. The data are presented as means $\pm$ S.D.. N = 6 mice in each group.

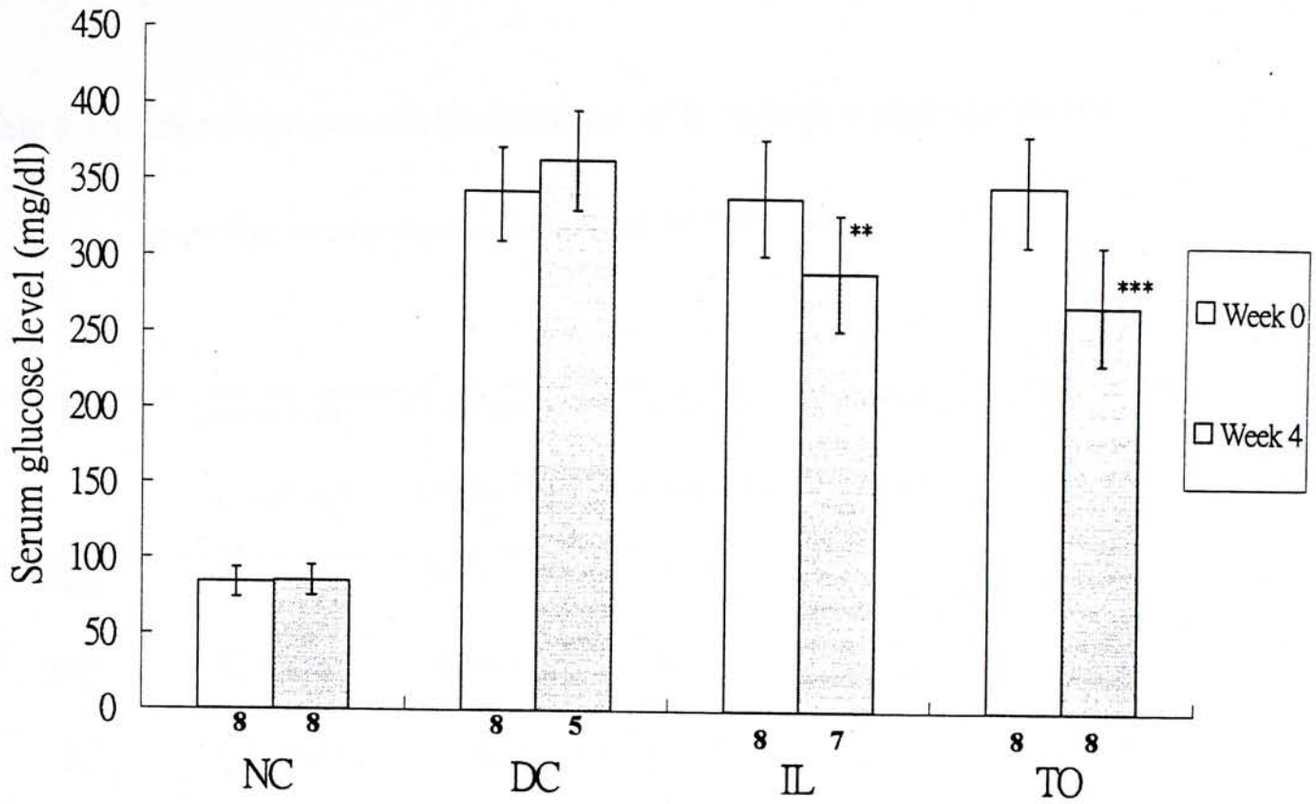

Figure 3.3.7 Effect of repeated administrations of IL on serum glucose level in streptozotocin-induced diabetic mice

NC: Normal control, DC: Diabetic control, IL : *Ilex latifolia* extract (3g/kg)

TO: Tolbutaimde (50mg/kg)

Mice received IL, water or tolbutamide daily for 4 weeks. Glucose concentration was determined as described in Materials and Methods. All values are presented as means ± S.D. of 5-8 mice. The number of mice of each group is shown at the bottom of each bar.

** $p < 0.01$, *** $p < 0.005$, significantly different from the diabetic control by Student's t-test.

Table 3.3.1 Effects of repeated administrations of IL on body weight change and mortality in streptozotocin-induced diabetic mice

	Initial body weight (g)	Final body weight (g)	% body weight gain	Number of deaths	Mortality (%)
NC	21.8±0.9	24.5±1.1	12.4	0	0
DC	21.9±1.0	15.2±1.2	-30.1	8	36.3
IL	21.7±0.8	17.4±0.9	-19.6	4	18.2
TO	21.6±0.8	19.1±1.5	-11.4	0	0

NC: Normal control, DC: Diabetic control, IL: *Ilex latifolia* extract(3g/kg),

TO: Tolbutamide (50mg/kg)

Mice received water, IL or tolbutamide for 4 weeks. Body weight was recorded before and after the experimental period for all three sets of experiments. Mortality was calculated as the number of deaths divided by the total number of treated animals (22) in all three sets of experiments.

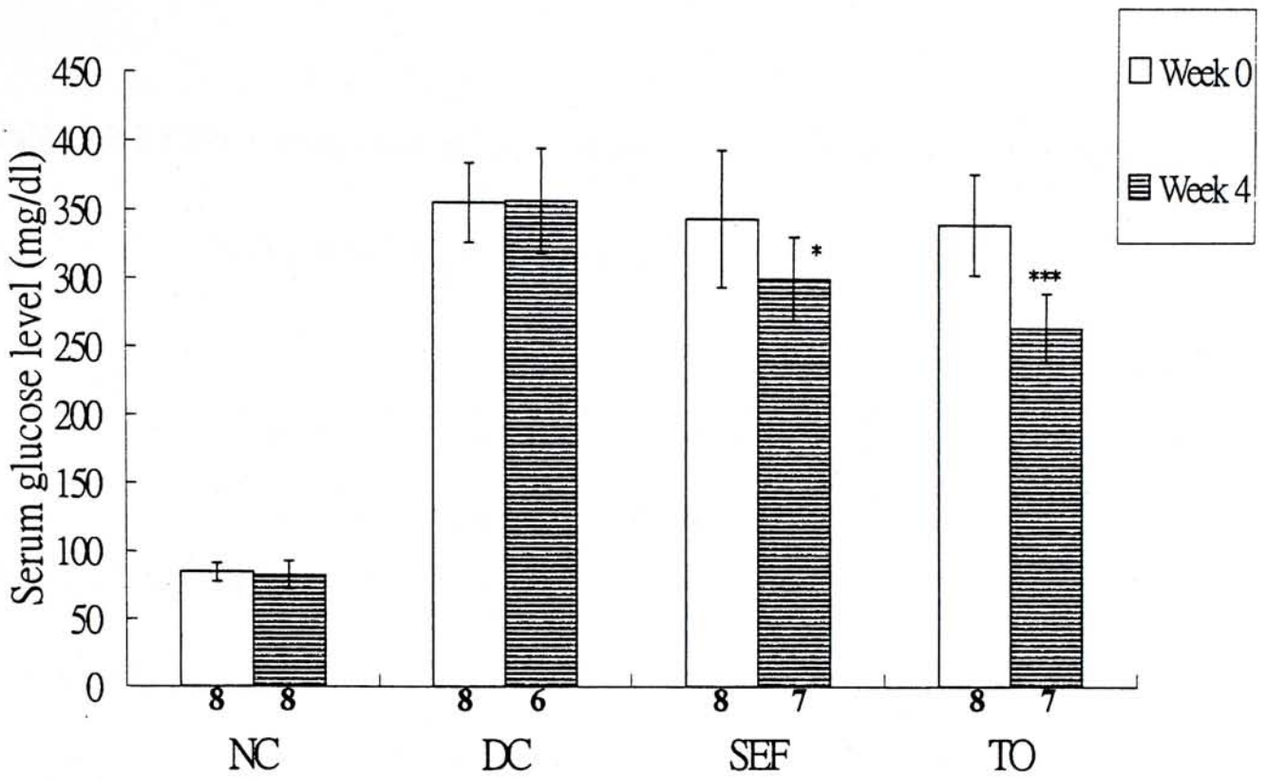

Figure 3.3.8 Effect of repeated administrations of SEF on serum glucose level in streptozotocin-induced diabetic mice

NC: Normal control, DC: Diabetic control,

SEF: Saponin-enriched fraction (100mg/kg), TO: Tolbutamide (50mg/kg)

Mice received SEF, 5% ethanol, or tolbutamide daily for 4 weeks. Glucose concentration was determined as described in Materials and Methods. All values are presented as means ± S.D. of 6-8 mice. The number of mice in each group is shown at the bottom of each bar.

*$p < 0.05$, *** $p < 0.005$, significantly different from the diabetic control by Student's t-test.

Table 3.3.2 Effects of repeated administrations of SEF on body weight change and mortality in streptozotocin-induced diabetic mice

	Initial body weight (g)	Final body weight (g)	% body weight gain	Number of deaths	Mortality (%)
NC	21.5±0.7	24.5±1.2	12.4	0	0
DC	21.9±0.9	14.8±0.9	-30.1	6	27.3
SEF	21.7±0.8	17.2±0.9	-19.6	1	4.5
TO	21.6±1.3	18.2±1.3	-11.4	1	4.5

NC: Normal control, DC: Diabetic control,

SEF: Saponin-enriched fraction (100mg/kg), TO: Tolbutamide (50mg/kg)

Mice received water, SEF or tolbutamide for 4 weeks. Body weight was recorded before and after the experimental period for all three sets of experiments. Mortality was calculated as the number of deaths divided by the total number of treated animals (22) in all three sets of experiments.

3.4 Discussion

The results of the present study demonstrated that both IL and SEF exerted a hypoglycemic effect in normal mice. Hypoglycemia could be observed only at the highest dose of IL and SEF. The glucose lowering effects of IL (3g/kg) and SEF (100mg/kg) were similar as shown in Figures 3.3.1. and 3.3.2. However IL had a higher saponin content (about 600mg saponin in 3g IL) than SEF. It indicated that the glucose lowering effect of saponin was higher than that of IL. However neither IL nor SEF exhibited a hypoglycemic effect in streptozotocin-induced diabetic mice. Theoretically, the blood or serum glucose level after an oral glucose load depends on factors like intestinal motility, glucose absorption, insulin secretion, metabolic factors or glucose utilization (Penchom *et al.*, 1995).

The mechanism of acute hypoglycemic action of IL and SEF may not be due to changes in insulin secretion. The reason is that when comparing the effects of IL or SEF in normal mice and diabetic mice, it was found that they produced hypoglycemic effects only in normal mice. If they could stimulate insulin secretion, they would also have lowered the serum glucose level in both normal and diabetic mice. Although the mechanism of this hypoglycemic action is still unknown, the glucose lowering effect of other saponins may give us a clue on the mechanism of IL or SEF. Tea extracts inhibits intestinal absorption of glucose and sodium in rats

(Sawsan *et al.*, 1994). Besides saponins can also affect gut permeability and active nutrient transport (Johnson *et al.*, 1986). One characteristic action of saponin is to induce hemolysis. It is because most saponins are highly surface-active, and many form addition complexes with sterols, including those associated with the plasma membranes of fungal, plant and animal cells. This leads to membrane destabilization and cell lysis (Bondi *et al.*, 1973). From these characteristics of saponins, one possible mechanism of IL or SEF on lowering serum glucose level may be due to its inhibition of intestinal glucose absorption.

The driving force for active glucose transport in the gut is provided by the presence of an electrochemical gradient for sodium across the brush-border membrane. It is maintained by active sodium extrusion at the basolateral membrane. The activity of this electrogenic pump gives rise to the substrate –dependent transmural potential difference. The effect of saponin is to bind cell membrane of gut and then reduce the permeability barrier to sodium at the brush border (such as inhibition of Na^+-K^+-ATPase, the enzyme for maintaining sodium gradient). The electrochemical gradient can be discharged and then the driving force for glucose transport can be removed. Moreover saponins may interact with the cell membrane and cause structural lesions. It would increase the turnover rate of intestinal mucosal cells, thus increasing the nutrient loss. Therefore IL or SEF may lower the serum

glucose level through this mechanism. However the effects of saponin on the electrochemical gradient on the cell membrane in gut had no effect on the serum glucose level. Therefore they had no effect on serum glucose level in diabetic mice.

The streptozotocin-induced diabetic mice is a model of insulin-dependent diabetes mellitus (IDDM). Streptozotocin selectively destroys the pancreatic cells that secrete insulin, which causes less active pancreatic cells and produces diabetes mellitus (Like *et al.*, 1990). Tolbutamide is an anti-diabetic drug. It can stimulate the remaining pancreatic cells in diabetic mice to secrete insulin to lower the blood glucose level. Therefore it was used as a positive control.

Moreover, the effects of repeated administrations of IL and SEF on the serum glucose level in diabetic mice were also studied. Both IL and SEF could reduce serum glucose level. However, the effects of saponin on the electrochemical gradient cannot be used to explain this phenomenon. The hypoglycemic effect of repeated administrations of IL or SEF may be due to other mechanisms. The research of Glombitza *et al.* (1993) on the hypoglycemic effects on *Zizyphus*s, a plant commonly used in Egyptian folk medicine for the treatment of different diseases, may give some hints which may be used to explain the effect of IL or SEF. The results indicated that the saponin of *Zizyphus* not only decrease the serum glucose level, but also reduces hepatic glucose-6-phosphate phosphatase activity. Some saponins from

fengugreeh seeds have been reported to decrease glucagon level (Valette *et al.*, 1984). One possible mechanism is that the glucagon lowering effect may enhance glucose utilization as indicated by increased hepatic glycogen, plasma pyruvate production and the decreased plasma glucose, hepatic phosphorylase, and glucose-6-phosphatase activities. Glycogen degradation can be rapidly stimulated by the direct hepatic action of different hormones. After that, glucagon exerts its glycogenolytic action through activation of adenyl cyclase leading to an increase in the level of cAMP (Extron *et al.*, 1972), a modulator of insulin secretion (Ferrel *et al.*, 1979) and induce insulin secretion. Moreover decreasing glucose-6-phosphatase would reduce the level of glucose-6-phosphate, which can be used to activate glycogen synthetase. Then glycogen synthetase can be induced to decrease the serum glucose level. Besides, IL contains, in addition to saponin, many different components such as polyphenol or urosolic acid as mentioned in Chapter 1. Polyphenol can also inhibit cAMP phosphodiesterase (Ferral *et al.*, 1979). Therefore an elevated cAMP level can induce insulin secretion to lower the serum glucose level. It indicated that the hypoglycemic effect of repeated administrations of IL and SEF may involve more than one mechanism. Both IL and SEF contain many different components. It is difficult to conclude that the hypoglycemic effect of IL and SEF is due to a particular component. Therefore IL and SEF should be further purified to find out the

component(s) with hypoglycemic effect. On the other hand, the effect of IL and SEF on metabolic enzymes such as glucose-6-phosphatase can be tested to find out its mechanism.

Chapter 4 Hypolipidemic effect of *Ilex latifolia* extract and its saponin-enriched fraction

4.1 Introduction

4.1.1 High serum cholesterol and triglyceride as a risk factor of CHD

4.1.2 Diabetes induces hypercholesterolemia and hypertriglyceridemia

4.1.3 Hypolipidemic effect of tea

4.1.4 Hypolipidemic effect of *Ilex latifolia* and Saponin

4.1.5 Objectives

4.2 Materials and Methods

4.2.1 Materials

4.2.2 Effect of repeated administrations of IL and SEF in normal mice fed with a high-cholesterol emulsion

4.2.3 Effect of repeated administrations of IL and SEF on serum cholesterol level and triglyceride level in hyperlipidemic mice

4.2.4 Effect of repeated administrations of IL and SEF on serum cholesterol and triglyceride level in diabetic mice

4.2.5 Determination of serum triglyceride and cholesterol levels

4.3 Results

4.3.1 Effect of repeated administrations of IL and SEF on serum cholesterol level in normal mice fed with a high-cholesterol emulsion

4.3.2 Effect of repeated administrations of IL and SEF on serum triglyceride level in normal mice fed with a high-cholesterol emulsion

4.3.3 Effect of repeated administrations of IL and SEF in hyperlipidemic mice

4.3.4 Effect of repeated administrations of IL and SEF on serum cholesterol level and triglyceride level in diabetic mice

4.4 Discussion

4.1 Introduction

4.1.1. High serum cholesterol level and triglycerides as a risk factor of Coronary Heart Disease (CHD)

A major medical disorder of the twentieth century is CHD. CHD accounts for over 260,000 deaths in the UK a year: more than four out of ten of all deaths (British Heart Foundation Health Promotion Research Group, 1999). There are many risk factors for CHD, such as smoking, diet and obesity. The concept of risk factors constitutes a major advance for developing strategies for preventing CHD. One of them is the serum cholesterol level. During the past 40 years, researchers have discovered a strong relationship between high concentration of serum cholesterol and CHD (Lipid Research Clinics Program, 1984). It may be due to the large amount of serum cholesterol that accumulates in the subintimal layer of the arteries to form plaques. The plaques are usually associated with degenerative changes in the arterial wall. They often protrude through the intima into the flowing blood and the roughness of their surfaces causes blood clots to develop, with resultant thrombus formation to cause death.

Unlike serum cholesterol level, the relationship between CHD and serum triglyceride level is controversial. Hokanson *et al.* (1996) demonstrated that triglyceride is a risk factor for cardiovascular disease for both men and women in the

general population, independent of HDL cholesterol. Avins *et al.* (2000) observed that measurement of serum triglyceride level does not provide clinically meaningful information of CHD risk. National Institutes of Health Consensus Development Conference Statement (1992) indicated that the relationship between CHD and triglycerides cannot be concluded. Triglyceride-rich lipoproteins can be atherogenic. Furthermore, elevated triglycerides produce fibrinolytic activity, which may contribute over time to the atherosclerotic process.

*4.1.2 **Diabetes induces hypercholesterolemia and hypertriglyceridemia***

Clinical and epidemiological reports have confirmed that cardiovascular disease is the major cause of death in diabetic mellitus (Brain *et al.*, 1997). Hypercholesterolemia is a feature frequently observed in humans with diabetes mellitus and certainly contributes to the high prevalence of atherosclerosis and coronary heart disease associated with metabolic disorder (Pierre *et al.*, 1992). Cholesterol absorption is increased and the defect in the removal of lipoproteins through LDL receptors in diabetic mice may be the possible mechanism for the high cholesterol level (Shimida *et al.*, 1995). Besides, hypertriglyceridemia also occurs. It is because insulin can act on adipose tissue to promote triglyceride storage and inhibit intracellular lipolysis and increase hepatic triglycerides. A lack of insulin can

cause production of hepatic triglycerides and defective hydrolysis of serum triglycerides. Therefore it is important to control the blood levels of triglycerides and cholesterol in diabetic patients.

4.1.3 Hypolipidemic effect of tea

Tea is one of the most popular beverages in the Chinese society. Recently, tea has received much attention as a protective agent against CHD (Imai *et al.*, 1995). Increased consumption of green tea has been shown to be associated with decreased serum level of cholesterol and triglyceride (Imai *et al.*, 1995). Drinking green tea can affect the plasma lipoprotein level. Imai *et al.* (1995) showed that drinking green tea can increase the proportion of HDL-cholesterol level and decrease the proportion of LDL-cholesterol and VLDL-cholesterol. The effect of green tea on hypocholesterolemia may be due to the flavonoid content in green tea (Yang *et al.*, 1997).

4.1.4 Hypolipidemic effect of Ilex latifolia and Saponin

As mentioned in Chapter 1, one of the effects of Kudingcha is hypocholesterolemia. However supporting scientific evidence is lacking. Li *et al.* (1996) showed that Kudingcha could decrease serum triglycerides and cholesterol

level in hypercholesterolemic mice. Therefore IL may also demonstrate a hypocholesterolemic effect.

Natural and synthetic saponins inhibit cholesterol absorption and reduce plasma cholesterol in experimental animals and are therefore of potential pharmacological utility in the treatment of hypocholesterolemia. Saponins are widely distributed in nature, forming a heterogeneous group of triterpenoid saponins and steroid glycosides of diverse biological activities that are themselves poorly absorbed from the intestinal tract, and interact with sterols in the intestinal lumen to prevent sterol absorption and hence lower the cholesterol level (Harwood *et al.*, 1993). Many triterpenoid glycosides (saponins) were discovered in different types of Kudingcha (Nakanishi *et al.*, 1981; Ouang *et al.*, 1998). Moreover two kudingosides can be found in Kudingcha (*Ligustrum pedunculare Rhed*) as an inhibitor of acyl-CoA: cholesterol acyltransferase (ACAT) which catalyzes intracellular esterification of cholesterol. Therefore the saponins in Kudingcha may also reduce serum cholesterol level through cholesterol esterification.

4.1.5 Objectives

Although the effect of IL on serum cholesterol level has been studied, the effect of IL and SEF is still unknown. Therefore the effect of IL at different concentrations

(0.3g/kg, 1g/kg and 3g/kg) on the serum triglyceride level and serum cholesterol level in mice with hypercholesterolemia and hypertriglyceridemia were studied. Besides the effect of SEF was determined. The effect of IL and SEF on serum cholesterol and triglyceride levels in diabetic mice was also examined.

4.2 Material and Methods

4.2.1 Materials

Nicotinic acid, sodium hydroxide, hydrochloric acid, cholesterol, sodium cholate and sucrose were obtained from Sigma Chemical Co. Olive oil and lard were obtained from a local supermarket.

4.2.2. Effect of repeated administrations of IL and SEF in normal mice fed with a high-cholesterol emulsion

Eight to twelve week-old male Balb/c mice were housed in an animal room on a 12h light/dark cycle (light: 0600-1800) period in LASEC of the Chinese University of Hong Kong. They were randomly divided into four groups and were fed a high-cholesterol emulsion with different drugs. The method described by Morishita *et al.* (1986) was modified and used to prepare the high-cholesterol emulsion. Three groups of mice (the experimental group) were fed a high-cholesterol emulsion (cholesterol 4.5g, cholic acid sodium salt 1.5g, sucrose 50g, lard 60g, distilled water 39ml) daily at noon for 4 weeks. IL at different concentrations were orally administered daily for 4 weeks two hours after administration of a high-cholesterol emulsion. For the positive control, nicotinic acid was orally administered instead of IL. Water instead of IL was administered into negative control. The normal control

group was treated with a control emulsion (sucrose 56g, olive oil 60g and water 39 ml) and water. The experiment to investigate the effect of SEF was the same as that of IL. Blood samples were collected before the commencement of the experiment, and again on day 15, day 22 and day 29 after overnight fasting. The mice had free access to food and water during the experimental period.

4.2.3 Effects of repeated administrations of IL or SEF on serum cholesterol level and triglyceride level in hyperlipidemic mice

Mice were fed with a high-cholesterol emulsion for two weeks to elevate the serum cholesterol and triglyceride levels. IL or SEF was administered daily for 4 weeks after hyperlipidemia had been achieved. Blood was taken before and after administration of the samples to determine the serum cholesterol and triglycerides levels.

4.2.4 Effects of repeated administrations of IL or SEF on serum cholesterol and triglyceride levels in diabetic mice

The streptozotocin-induced diabetic mice mentioned in Section 3.2.7 were used. Blood was taken before and after the experiment to determine serum cholesterol and triglyceride levels.

4.2.5 Determination of serum triglyceride and cholesterol level

Serum levels of triglyceride and cholesterol were determined by using the enzymatic kits from Sigma Chemical Co. For the determination of cholesterol, 0.01ml serum was mixed with cholesterol reagent from Sigma and the mixture was incubated in water bath at 37°C for 10 min. Water was used as the blank. Absorbance was read at 500nm and the serum cholesterol level can be calculated. For the determination of triglyceride, 10 μl serum was mixed with triglyceride reagent from Sigma and the mixture was incubated for 5 min at 37°C. Water was used as the blank. Absorbance at 540nm was read to determine the serum triglyceride level.

4.3. Results

4.3.1 Effect of repeated administrations of IL and SEF in serum cholesterol level normal mice fed with high-cholesterol emulsion

The effect of IL at different concentrations (0.3g/kg, 1g/kg and 3g/kg) on the cholesterol level is shown in Figure 4.3.1. The effect of SEF is shown in Figure 4.3.2. The serum total cholesterol level in the mice was monitored throughout the experiment.

Before cholesterol feeding, no difference was observed between the serum cholesterol levels of both normal and high cholesterol groups. After 14 days of feeding a high-cholesterol emulsion, an increase in serum cholesterol level was observed in the treated groups. No change occurred in control group. It was found that IL at the highest dose (3g/kg) significantly reduced the cholesterol level by 12% at days 15, 22 and 29 (Figure 4.3.1). However the two lower doses of IL (0.3g/kg and 1g/kg) had no effect on serum cholesterol level at all time points. SEF showed a tendency to reduce serum cholesterol level at day 15, and also significantly lowered the cholesterol level by 9 % at days 22 and 29. Nicotinic acid, which was used as a positive control, lowered the serum cholesterol level in two sets of experiments (Figures 4.3.1 and 4.3.2) and showed a significant effect except at day 15 (Figure 4.3.2).

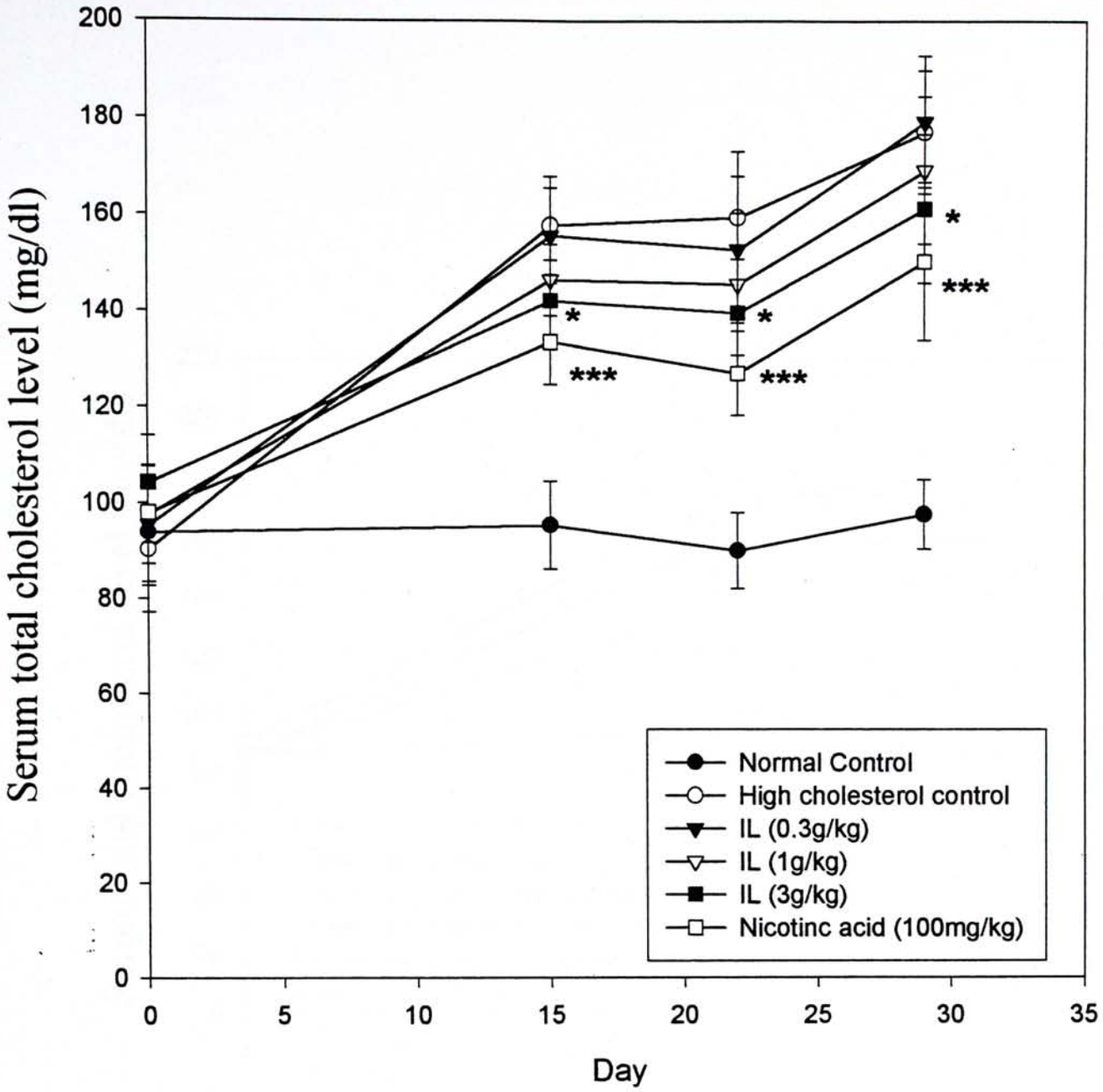

Figure 4.3.1 Effects of different concentrations of IL on serum total cholesterol level in mice fed a high-cholesterol emulsion. Mice received different concentrations of IL, water or nicotinic acid daily for 4 weeks. Serum total cholesterol concentrations were determined as described in Materials and Methods. Data are presented as means $\pm$ S.D.($n = 5$)

* $p < 0.05$, *** $p < 0.005$, significantly different from the high-cholesterol control by Student's t-test.

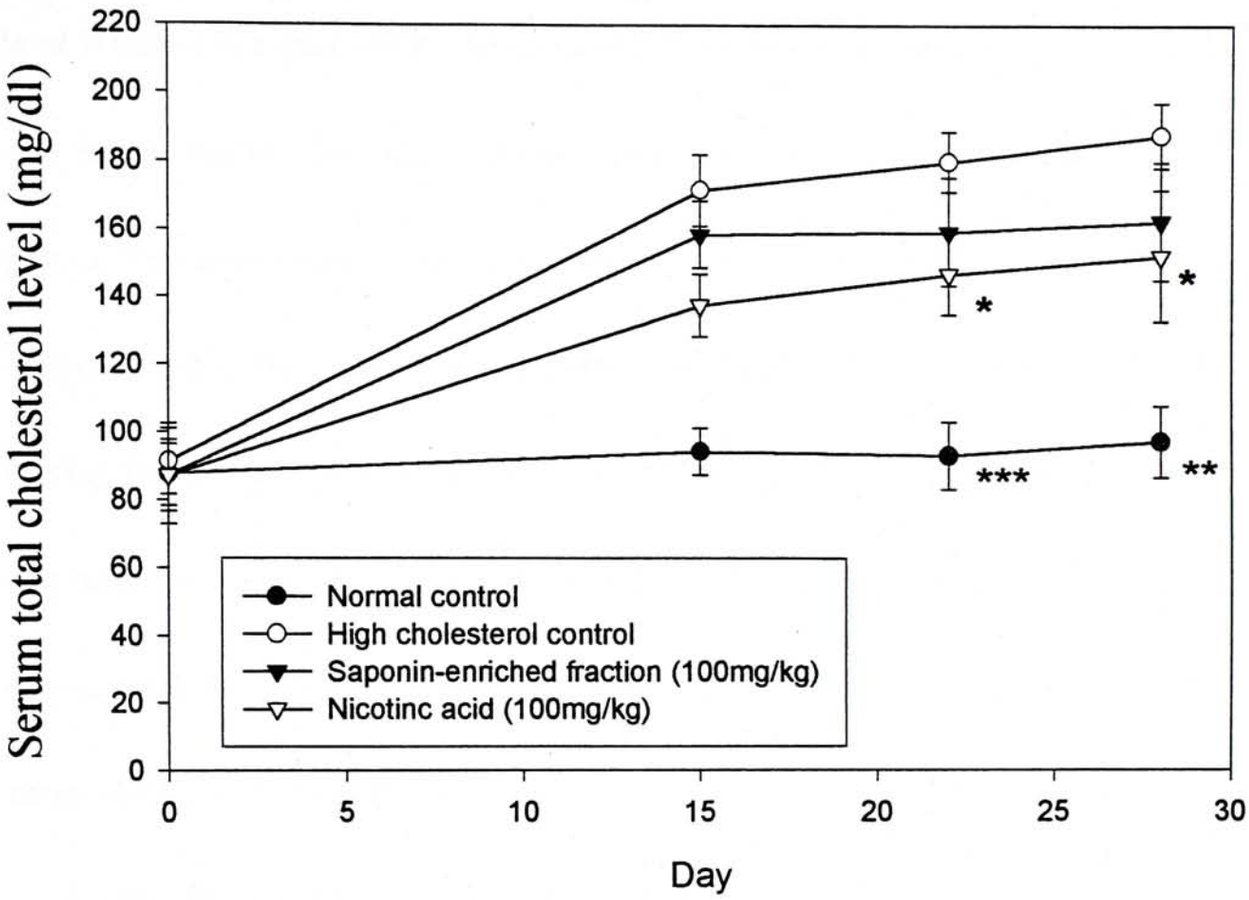

Figure 4.3.2 Effects of SEF on serum total cholesterol level in mice fed a high-cholesterol emulsion. Mice received SEF, nicotinic acid or 5% ethanol daily for 4 weeks. Serum total cholesterol were determined as described in Materials and Methods. Data are presented as means $\pm$ S.D. (n = 5).

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, significantly different from the high-cholesterol control by Student's t-test.

4.3.2 Effect of repeated administrations of IL and SEF in serum triglyceride level of normal mice fed with a high-cholesterol emulsion

The dose response curve of IL at different concentrations on serum triglyceride level is shown in Figure 4.3.3. The effect of SEF is shown in Figure 4.3.4.

Before feeding the high-cholesterol diet, there was no difference between all groups. The serum triglyceride level was elevated after two weeks of feeding and maintained at a high level thereafter when compared with the normal control. IL (3g/kg) brought a significant about 13% reduction of the serum triglyceride level. The other two doses (0.3g/kg and 1g/kg) did not affect serum triglyceride level. SEF significantly reduced the level of serum triglyceride at all time points after establishment of hypertriglyceridemia. Nicotinic acid, a positive control, also showed a reducing effect on the serum triglyceride level.

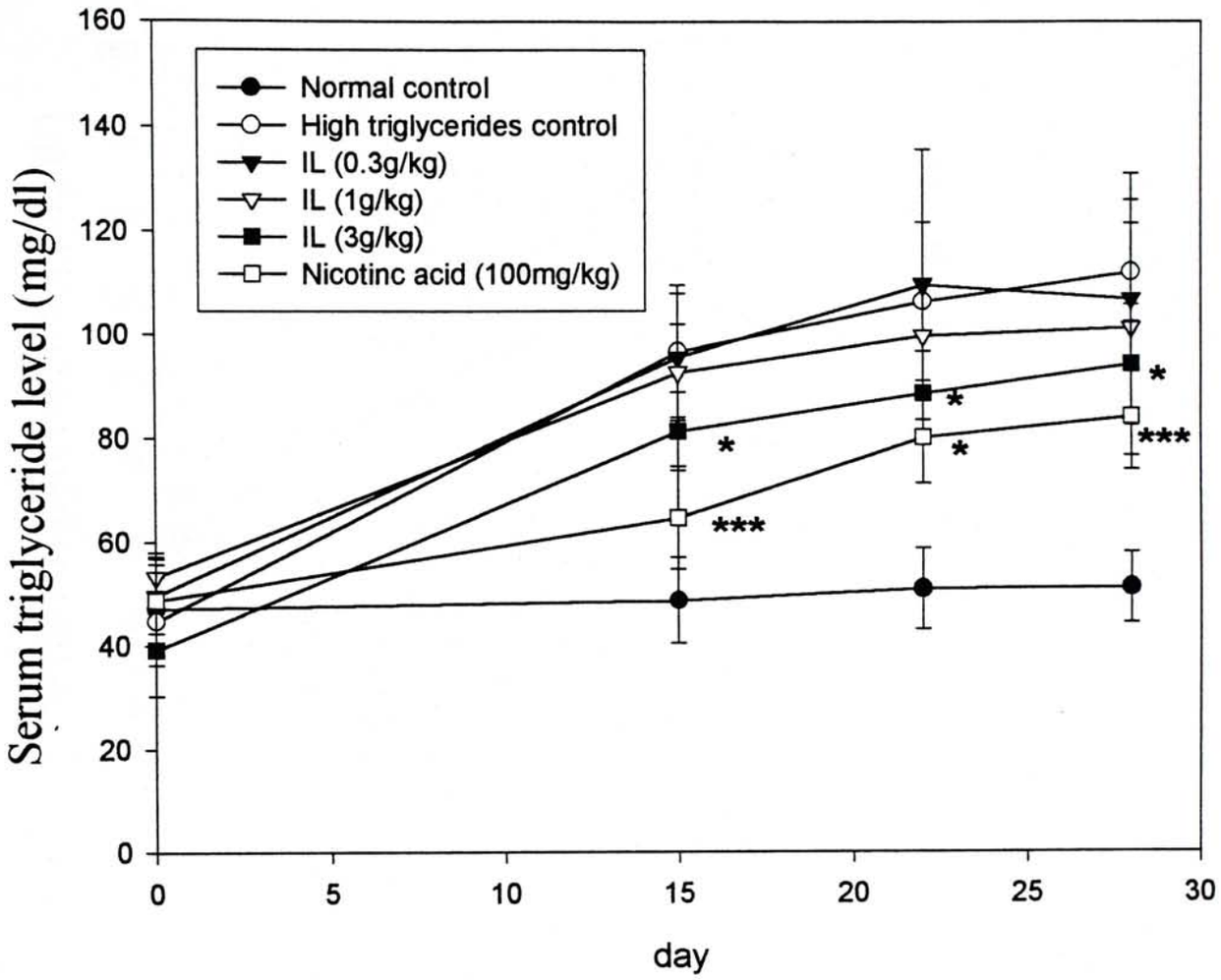

Figure 4.3.3 Effects of different concentrations of IL on serum triglycerides level in mice fed with a high-cholesterol emulsion. Mice received different concentrations of IL, water or nicotinic acid daily for 4 weeks. Triglyceride concentrations were determined as described in Materials and Methods. Data are presented as means $\pm$ S.D. (n = 5). * $p < 0.05$, *** $p < 0.005$, significantly different from the high cholesterol control by Student's t-test.

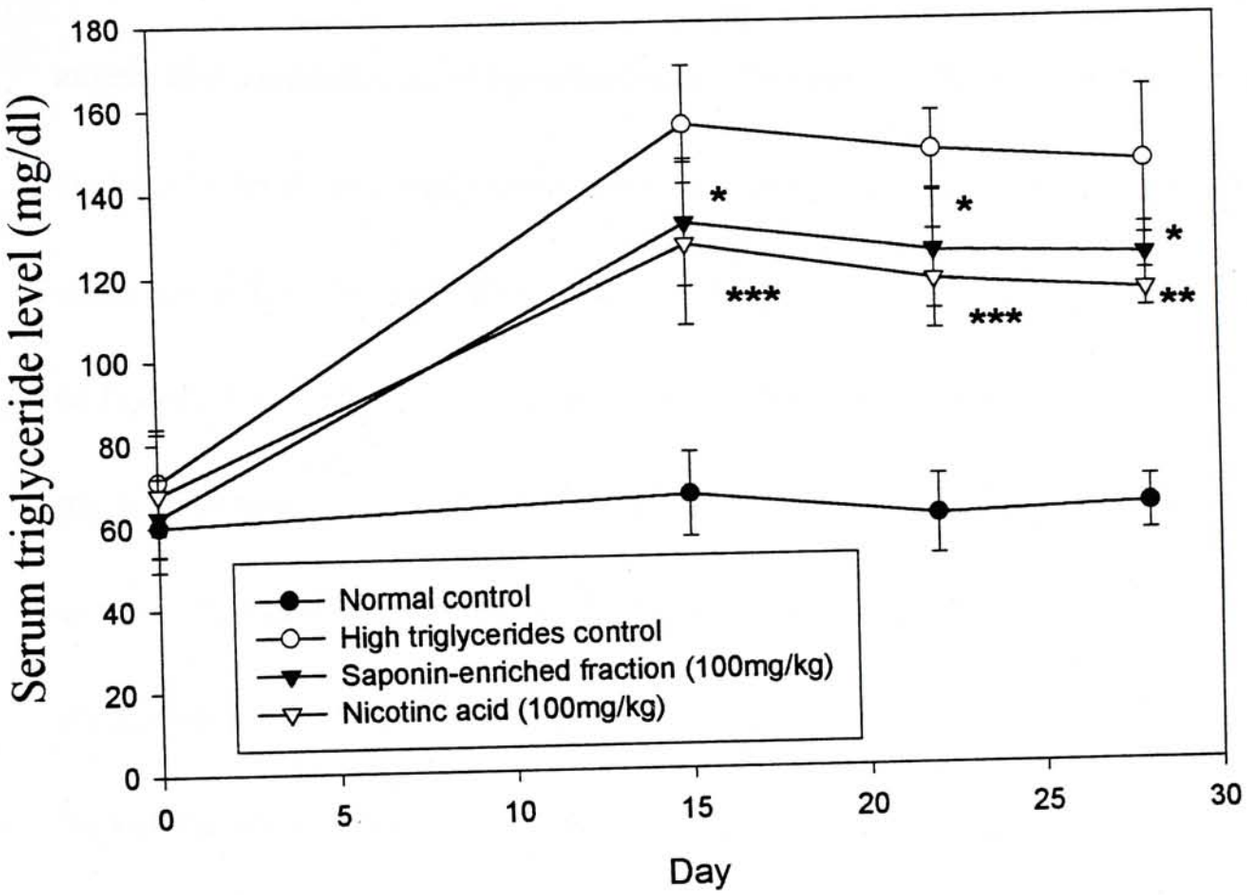

Figure 4.3.4 Effects of SEF on serum triglycerides level in mice fed a high-cholesterol emulsion. Mice received SEF, nicotinic acid or 5% ethanol daily for 4 weeks. Triglyceride concentrations were determined as described in Materials and Methods. Data are presented as means $\pm$ S.D. (n = 5).

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, significantly different from the high triglycerides control by Student's t-test.

4.3.3 Effect of repeated administrations of IL and SEF in the hyperlipidemic mice

Mice were first fed with a high-cholesterol emulsion to elevate the serum levels of cholesterol and triglyceride. Different samples (IL or SEF) were fed daily for one month after establishment of hyperlipidemia. The results of IL treatment on serum cholesterol level and triglyceride level are shown in Figures 4.3.5 and 4.3.6 respectively. The effects of SEF are shown in Figures 4.3.7 and 4.3.8. From the result of Figures 4.3.5 and 4.3.7, IL at the dose of 3g/kg, lowered serum cholesterol and triglyceride level. Compared with the high-cholesterol control and high-triglyceride control, IL significantly reduced the level of cholesterol by 11 % and serum triglyceride level by 7.5%. SEF also significantly reduced serum cholesterol level (15 %) and triglyceride level (15%). When the effects of IL and SEF are compared, both of them were found to have similar effects on reducing serum cholesterol and triglyceride levels.

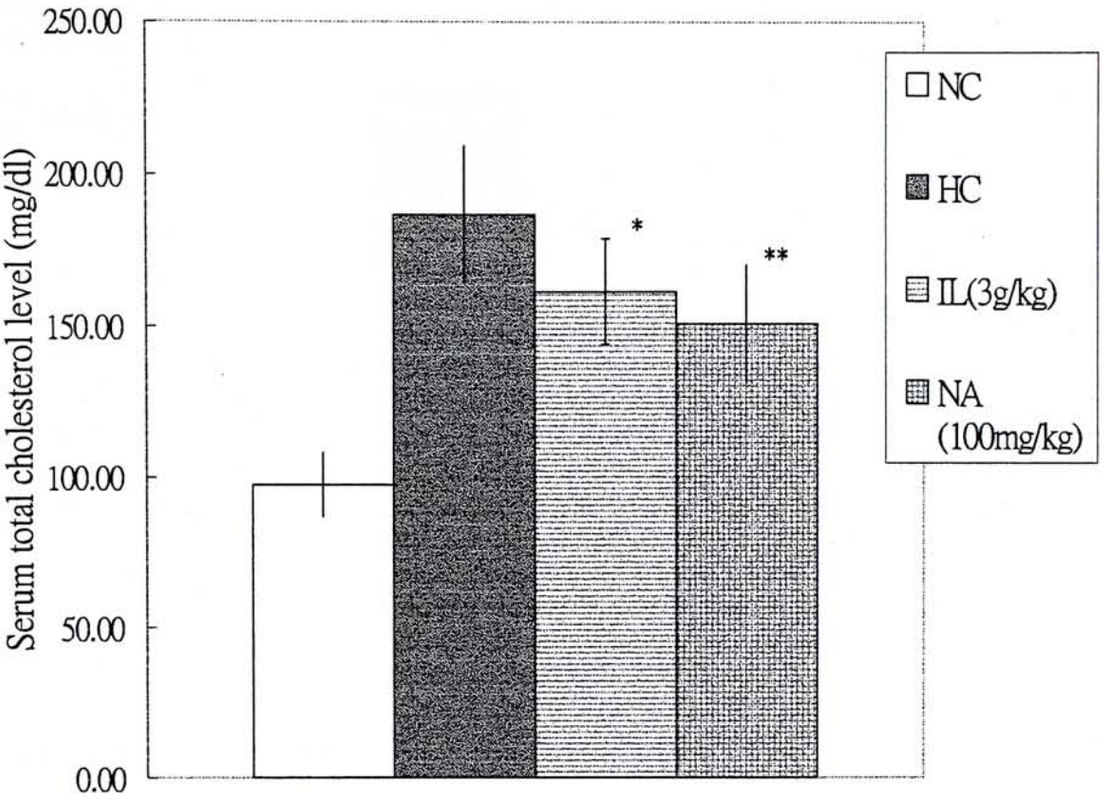

Figure 4.3.5 Effects of repeated administrations of IL on serum cholesterol level in hyperlipidemic mice.

Mice received IL, nicotinic acid (NA) or water for 4 weeks. Serum total cholesterol concentration was determined as described in Materials and Methods. Data are presented as means ± S.D. (n=5).

*$p < 0.05$, ** $p < 0.01$, significantly different from the high cholesterol control by Student's t-test

NC = Normal control, HC = High-cholesterol control

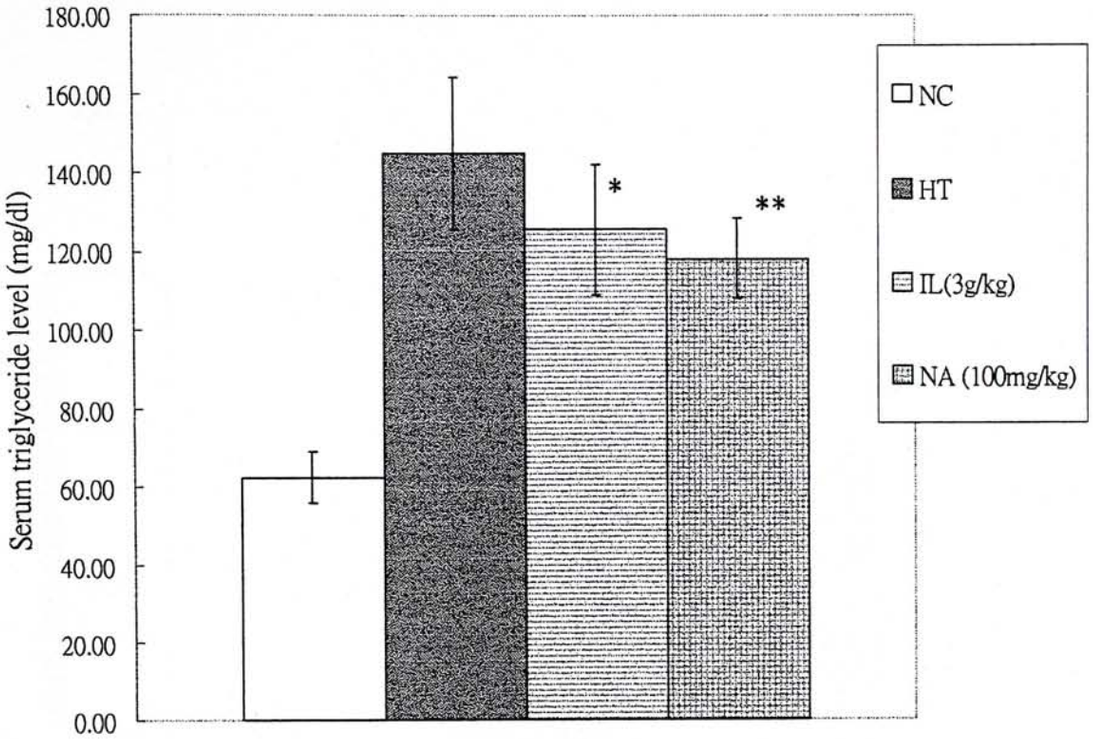

Figure 4.3.6 Effects of repeated administrations of IL on serum triglyceride level in hyperlipidemic mice.

Mice received IL, nicotinic acid (NA) or water for 4 weeks. Triglyceride concentration was determined as described in Materials and Methods. Data are presented as means ± S.D. (n=5).

*$p < 0.05$, ** $p < 0.01$, significantly different from the high-triglyceride control by Student's t-test

NC = Normal control, HT = High-triglyceride control

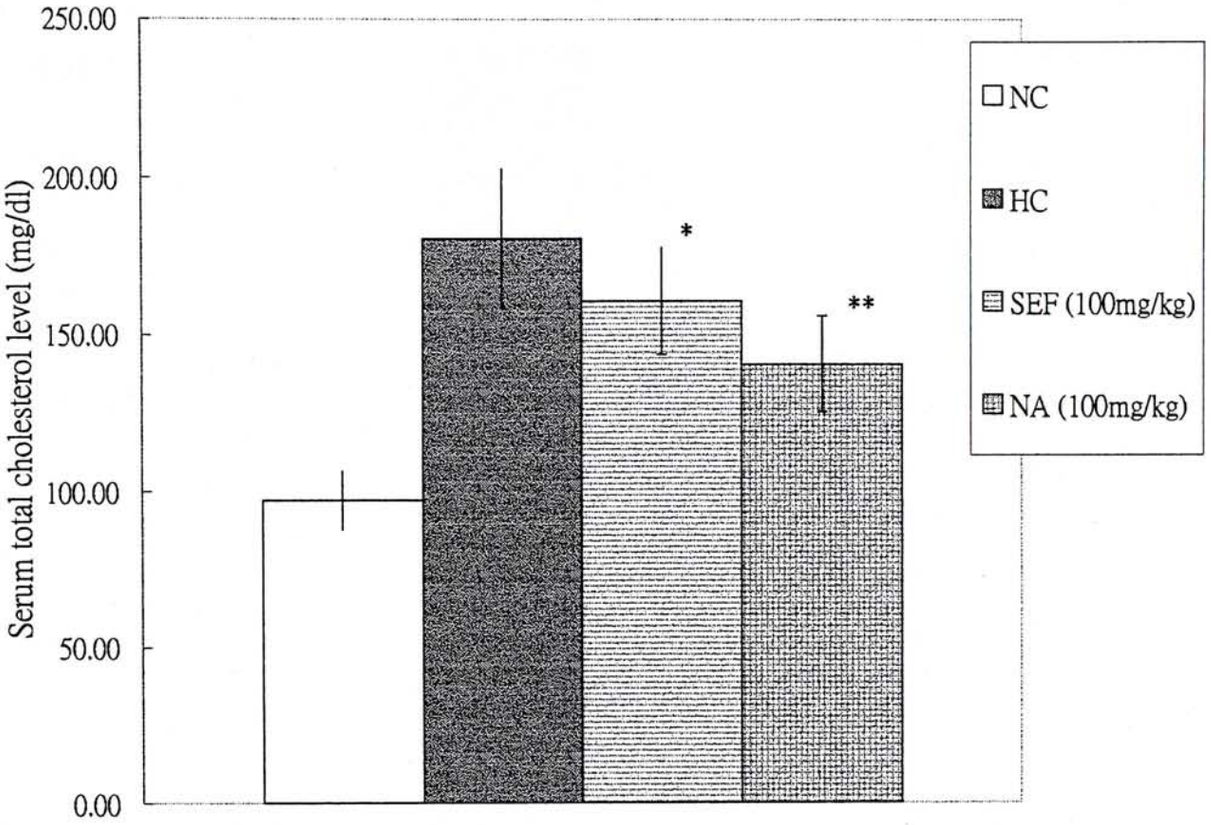

Figure 4.3.7 Effects of repeated administrations of SEF on serum total cholesterol level in hyperlipidemic mice.

Mice received SEF, nicotinic acid (NA) or water for 4 weeks. Serum total cholesterol was determined as described in Materials and Methods. Data are presented as means ± S.D. (n=5).

*$p < 0.05$, ** $p < 0.01$, significantly different from the high cholesterol control by Student's t-test

NC = Normal control, HC = High-cholesterol control

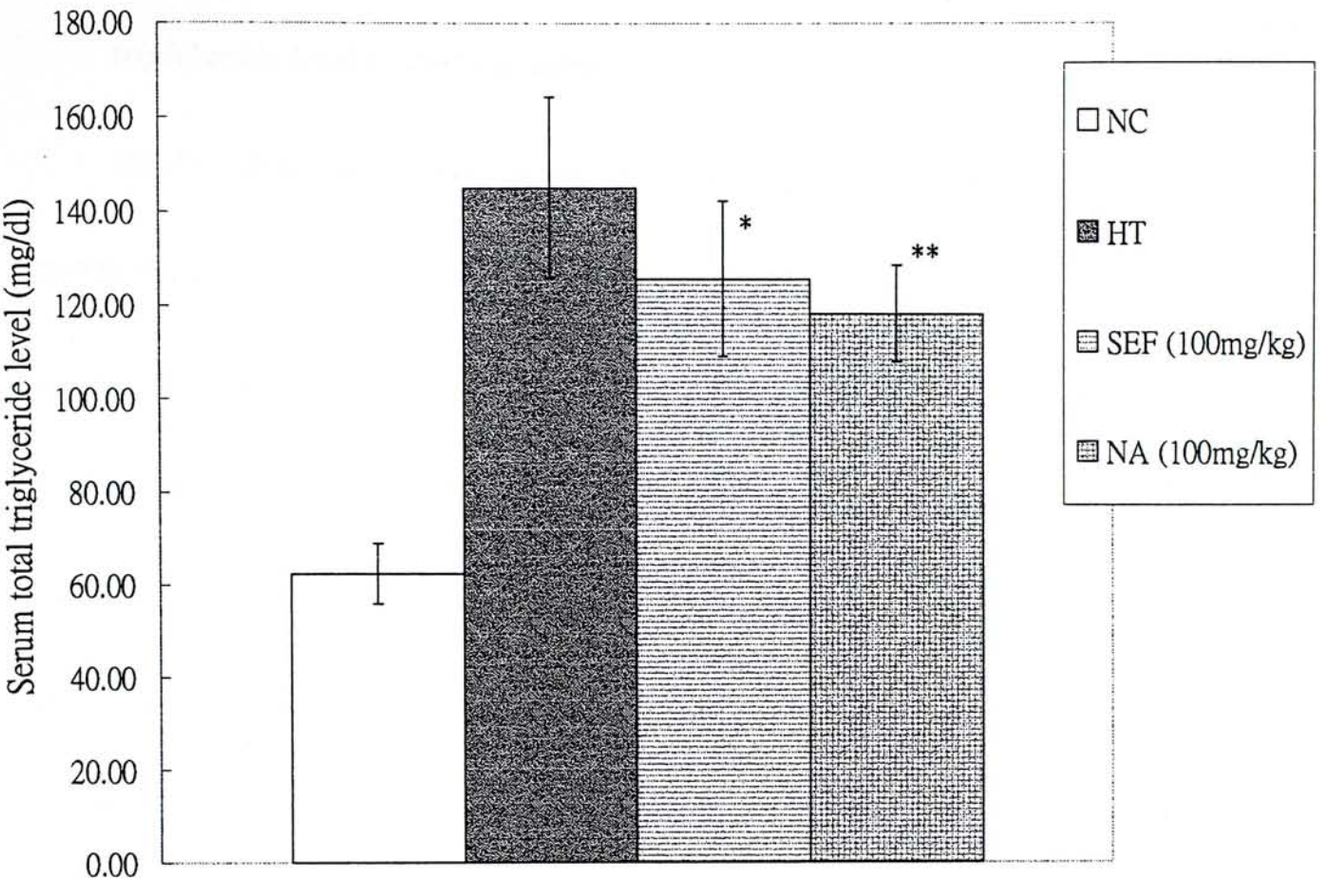

Figure 4.3.8 Effects of repeated administrations of SEF on serum triglyceides level in hyperlipidemic mice.

Mice received SEF, nicotinic acid (NA) or water for 4 weeks. Triglyceride concentration was determined as described in Materials and Methods. Data are presented as means ± S.D. (n=5).

*$p < 0.05$, ** $p < 0.01$, significantly different from the high-triglyceride control by Student's t-test

NC = Normal control, HT = High-triglyceride control

4.3.4 Effect of repeated administrations of IL and SEF on serum cholesterol and triglyceride level in diabetic mice

Insulin deficiency can cause disorders of triglyceride and cholesterol metabolism. Elevated serum cholesterol and triglyceride are found in diabetic animals. IL significantly reduced serum cholesterol level by 13% (Figure 4.3.9) and triglyceride level by 18% (Figure 4.3.10). SEF also significantly reduced serum levels of cholesterol by 13% and triglyceride by 16%. The effects of SEF on serum level of cholesterol and triglyceride are shown in Figures 4.3.11 and 4.3.12 respectively.

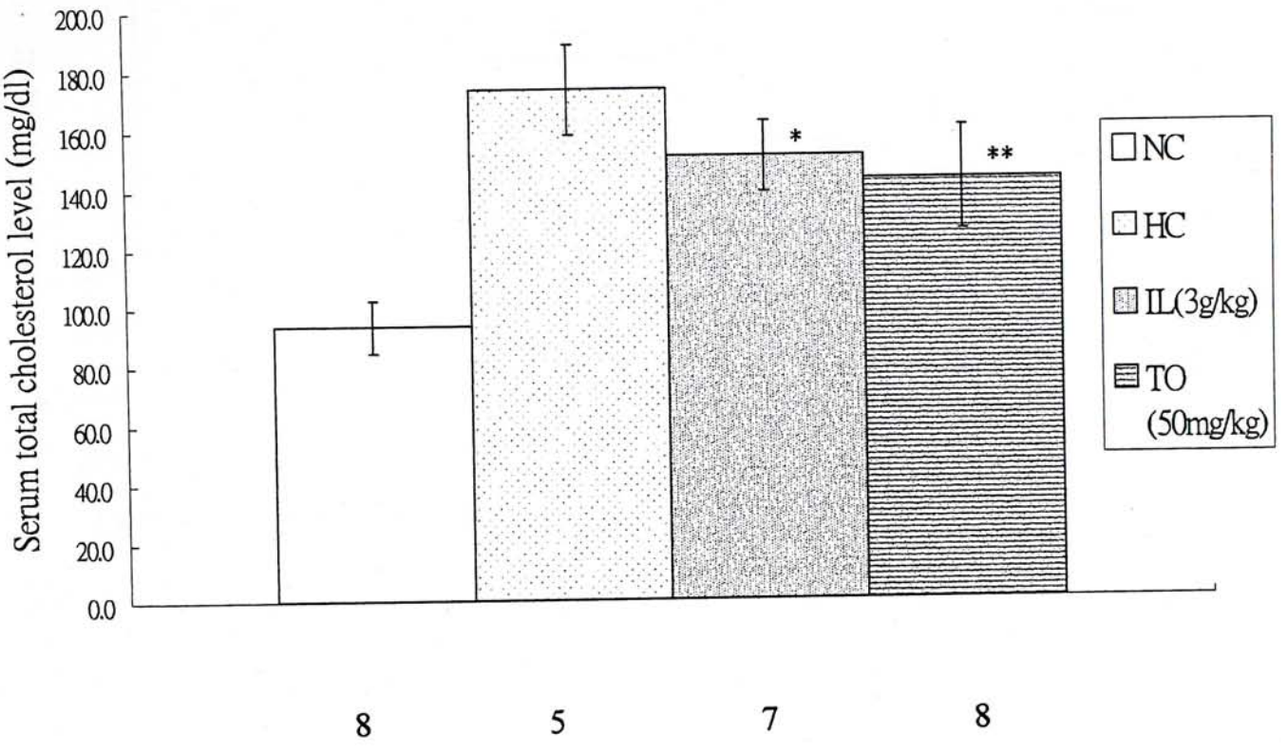

Figure 4.3.9 Effect of repeated administrations of IL on total serum cholesterol level in diabetic mice

NC: Normal control, HC: High cholesterol control, IL : *Ilex latifolia* extract (3g/kg)

TO: Tolbutamde (50mg/kg)

The number of mice in each group was indicated at the bottom of each bar. Mice received IL, water or tolbutamide daily for 4 weeks. Serum cholesterol concentrations were determined as described in Material and Methods. All values are presented as means ± S.D.

*$p < 0.05$, ** $p < 0.01$, significantly different from the high cholesterol control by Student's t-test.

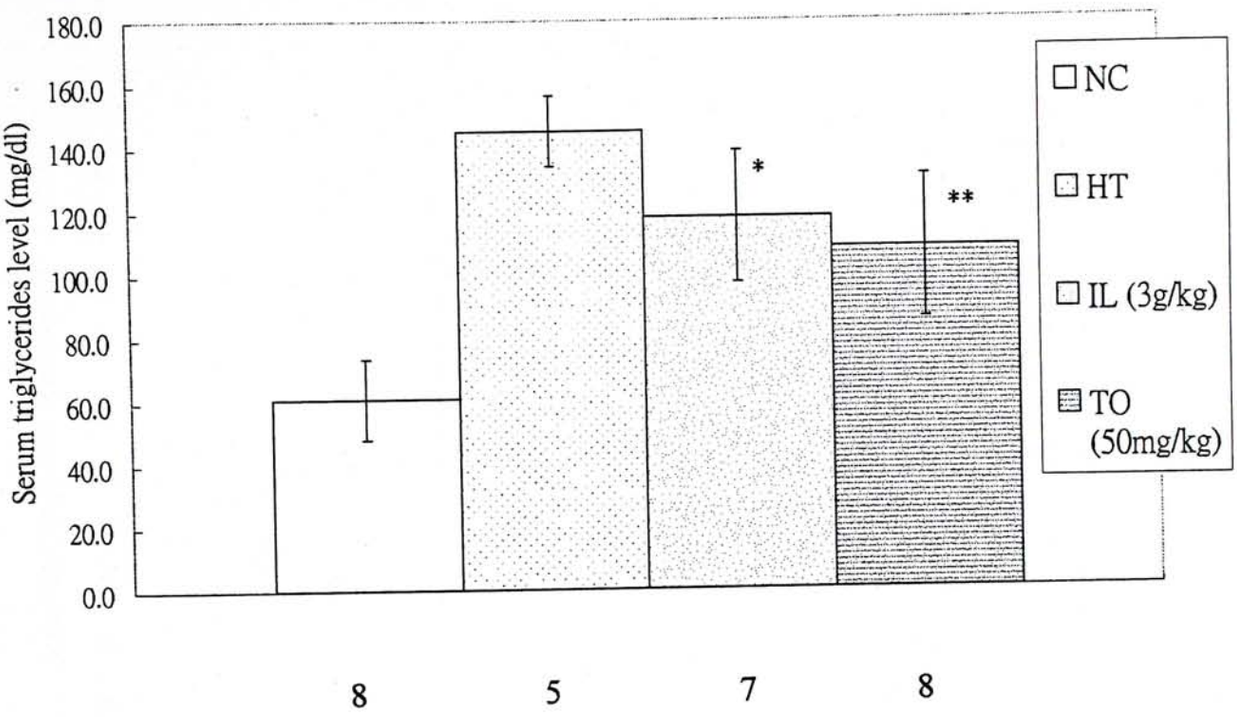

Figure 4.3.10 Effect of repeated administrations of IL on serum triglycerides level in diabetic mice

NC: Normal control, HT: High triglyceride control, IL : *Ilex latifolia* extract (3g/kg)

TO: Tolbutamide (50mg/kg)

The number of mice in each group was indicated at the bottom of each bar. Mice received IL, water or tolbutamide daily for 4 weeks. Serum triglyceride concentrations were determined as described in Materials and Methods. All values are represented as mean ± S.D.

*$p < 0.05$, ** $p < 0.01$, significantly different from the high triglycerides control by Student's t-test.

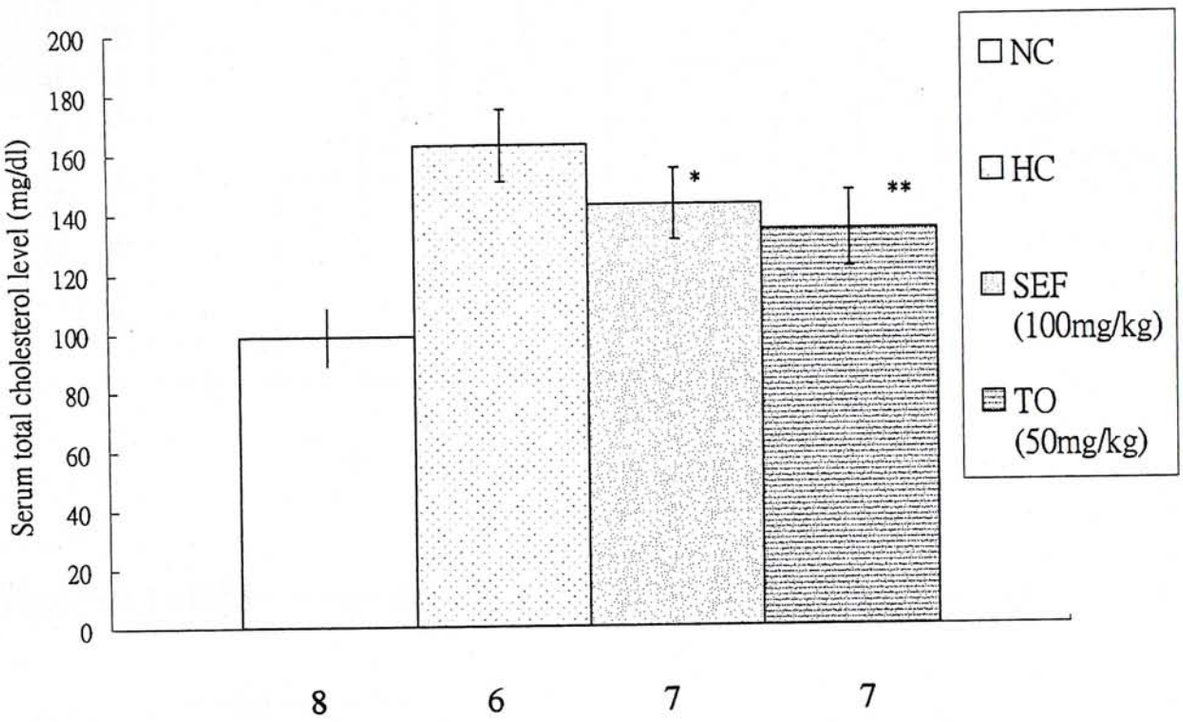

Figure 4.3.11 Effect of repeated administrations of SEF on serum total cholesterol level in diabetic mice

NC: Normal control, HC: High-cholesterol control,

SEF: Saponin-enriched fraction (100mg/kg), TO: Tolbutamide (50mg/kg)

The number of mice in each group was indicated at the bottom of each bar. Mice received SEF, 5% ethanol or tolbutamide daily for 4 weeks. Serum cholesterol concentrations were determined as described in Materials and Methods. All values are presented as means ± S.D.

*$p < 0.05$, ** $p < 0.01$, significantly different from the high-cholesterol control by Student's t-test.

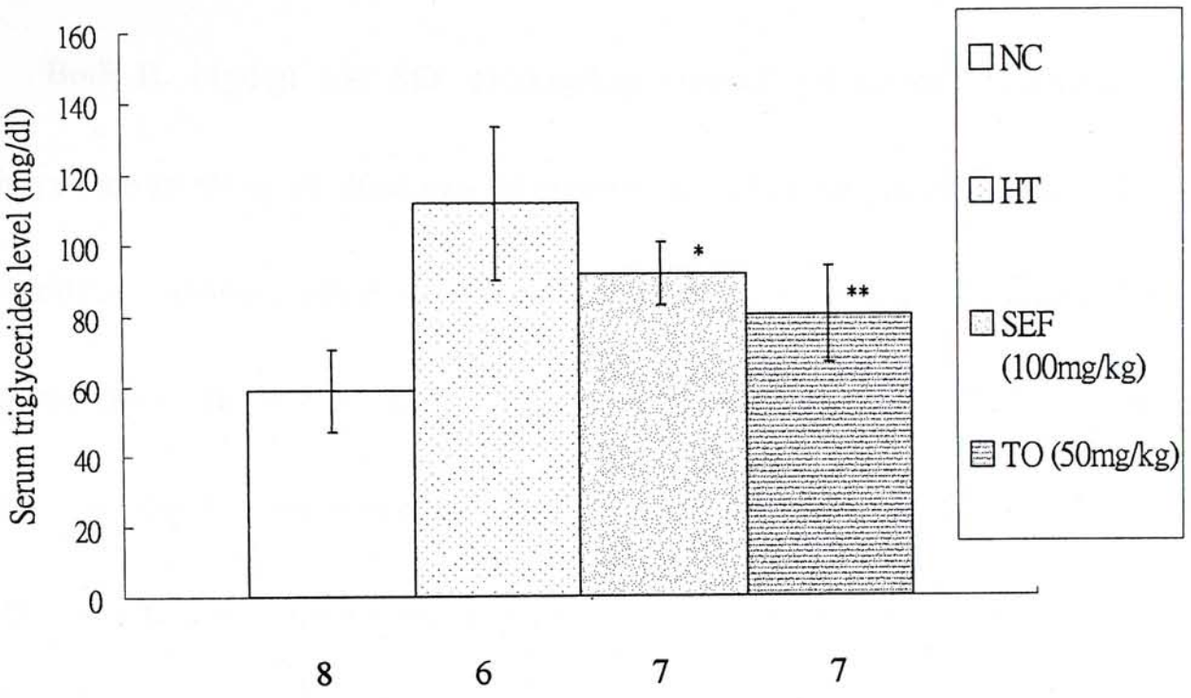

Figure 4.3.12 Effect of repeated administrations of SEF on serum triglyceride level in diabetic mice

NC: Normal control, HT: High-triglycerides control,

SEF : Saponin-enriched fraction (100mg/kg), TO: Tolbutamide (50mg/kg)

The number of mice in each group was indicated at the bottom of each bar. Mice received SEF, 5 % ethanol or tolbutamide daily for 4 weeks. Serum triglyceride concentration was determined as described in Materials and Methods. All values are presented as means ± S.D.

*$p < 0.05$, ** $p < 0.01$, significantly different from the high-triglycerides control by Student's t-test.

4.4 Discussion

Both IL (3g/kg) and SEF (100mg/kg) lowered the serum cholesterol and triglyceride levels in all three sets of experiments: effect on mice fed with a high-cholesterol emulsion, effect in hyperlipidemic mice and in streptozotocin-induced diabetic mice. The results regarding SEF and IL were similar. However IL had a higher saponin content than as mentioned in Chapter 1. It indicated that SEF may exert more potent hypolipidemic action than IL. It is because the presence of other components such as flavonoids in IL may interfere with the effect of saponin. Besides saponin is one major component in IL. Many studies have shown that saponin could reduce serum cholesterol level (Sidhu *et al.*, 1986; Harwood *et al.*, 1993; Morehouse *et al.*, 1999). The hypolipidemic effect of IL may therefore be due to the presence of saponin.

Results of a preliminary test (data not shown) indicated that, IL and SEF did not affect the serum cholesterol and triglyceride levels in hyperlipidemic mice after administration for two weeks. Both IL and SEF reduced the serum cholesterol and triglyceride level in mice fed with a high-cholesterol emulsion and hyperlipidemic mice. The effects of IL or SEF in mice fed with a high-cholesterol emulsion were faster than that of hyperlipidemic mice. Therefore IL or SEF may have an effect on gastrointestinal cholesterol absorption.

Both serum cholesterol and triglyceride levels in the streptozotocin-induced diabetic mice were reduced after administration of IL and SEF. The elevated serum cholesterol and triglyceride levels in diabetic mice are due to insulin deficiency. Insulin can affect serum cholesterol level by increasing cholesterol absorption (Masako *et al.*, 1995) and serum triglyceride level by stimulating storage of adipose tissue. Although the mechanism of IL or SEF is not known, IL and SEF may have an effect on insulin secretion as mentioned in the discussion of Chapter 3.

Serum cholesterol and triglyceride levels are reduced after consumption of green tea (Imai *et al.*, 1995). It may be due to the flavonoids present in green tea. Flavonids are polyphenolic compounds with a basic chemical structure of two aromatic rings joined by a three-carbon aliphatic chain. Several animal experiments have also demonstrated the effects of polyphenol in green tea in lowering serum cholesterol level (Muramatsu *et al.*, 1986; Chan *et al.*, 1999). IL also contains polyphenols, which may contribute in reducing the serum cholesterol and triglycerides levels.

Saponins form a heterogeneous group of triterpenes or steroid glycosides, which occur in hundreds of plant species (Oakenfull *et al.*, 1990). Many of these are staple items of the human diet (Oakenfull *et al.*, 1981). Examples particularly rich in saponins are soya bean (*Glycine maz*) and navy beans (*Phaseolus vulgaris*). Isolated

saponins and food containing saponins have been shown to lower plasma cholesterol concentration in a number of animal species (Malinow *et al.*, 1977). Although the mechanism of saponin in IL or SEF involved in reducing cholesterol is not known, the effect of other saponins in plants such as soya bean saponin can be used to deduce the mechanism of saponin in IL or SEF.

There appear to be several possible mechanisms by which saponin affects cholesterol metabolism. The most obvious and of the longest standing is that, because saponin can form complexes with cholesterol *in vitro*, they can also form similar complexes with cholesterol in the gut lumen and thus directly inhibit absorption of cholesterol (Coulson *et al.*, 1960; Jon *et al.*, 1984).

Another possible mechanism is that saponin interferes with the enterohepatic circulation of bile acids to affect cholesterol metabolism indirectly. Bile acids and their salts are relatively hydrophilic cholesterol derivatives that are synthesized in the liver. They pass through the bile duct into the small intestine where they promote lipid digestion and absorption by forming mixed micelles with fatty acids, monoglycerides and cholesterol. Lipid absorption occurs in the upper small intestine but the liberated bile acids are, for the most part, not absorbed until they reach the distal small intestine. Then they pass into the portal blood stream through an active transport process and then return to the liver for reuse. The reabsorption of bile acids

depends on the amount of liberated bile acids. Non-absorbable materials that bind bile acids make them pass on into the colon and eventually excreted in the faeces. The loss by faecel excretion can induce an increase in endogenous cholesterol synthesis resulting in reduction of serum cholesterol level to compensate for the loss (Natalia *et al.*, 1985).

Some saponins have recently been shown to form large mixed micelles with bile acids (Sidhu *et al.*, 1986). They can have molecular weights of several millions (Sidhu *et al.*, 1986). It is because saponin and bile acids are both amphiphilic compounds, partly hydrophobic and partly hydrophilic. In aqueous solution they form small micelles with their hydrophobic triterpene or steroid groups stacking together. When the two types of compounds are mixed, their hydrophobic groups interleave with each other in their stacks. The stacks become greatly extended, incorporating many hundreds of molecules. The large stacks indeed block reabsorption of bile acids from the small intestine (Sidhu *et al.*, 1986).

Another possibility is that saponins may affect cholesterol metabolism by interacting with sterols in the brush border membranes of the intestinal mucosal cells (Johnson *et al.*, 1986). Hemolytic saponins significantly increase the permeability of isolated intestine *in vitro*. Similar effects might occur in intact animals. It might thus affect nutrient adsorption.

Moreover the decrease in serum cholesterol concentration may also be associated with the enzyme acyl-CoA: cholesterol acyltransferase (ACAT). ACAT functions mainly to esterify cholesterol and store it as cholesterol ester. Thus it plays a key role in the intestinal absorption of cholesterol (Largis *et al.*, 1989). Dietary cholesterol is esterified before it is assembled in chylomicrons and secreted into the lymphatic system. Therefore decrease in intestinal esterification of cholesterol by inhibiting ACAT can decrease serum cholesterol level. Fukuda *et al.* (1996) showed that an extract of kudingcha can inhibit ACAT activity.

The mechanism of saponin in reducing serum cholesterol level can also be used to explain its effect on serum triglyceride level. After administration in diet, bile salts can emulsify the triglycerides in the small intestine, forming mixed micelles for intestinal lipase degradation. Saponin which binds with bile acids can interfere micelle formation and reduce the effect of intestinal lipase resulting in reduced absorption of triglyceride.

Chapter 5 Conclusion

IL is one of hollies in Trilex tea and is a kind of Kundingcha. SEF is one of the major components in IL. The acute and sub-chronic effects of IL and SEF were studied in mice. It was found that IL and SEF had no effect on serum AST and ALT activities in mice one day and two weeks after administration, indicating no hepatocyte damage after IL or SEF treatment.

In the detoxification test, IL and SEF had no effect on MROD and EROD activities after administration for one day and two-weeks, indicating that IL and SEF did not affect some of the phase I enzymes. However IL induced liver GST activity except for the lowest dose (0.3g/kg). No GST induction effect could be found in mice treated with SEF in the two sets of experiments. Comparing the effects of IL and SEF on GST induction, it was revealed that the components of IL for GST induction might not be related to its saponin fraction. Moreover GST is one of phase II enzymes for removing xenobiotic metabolites. Therefore increased GST activity can enhance the process of detoxificiation. Therefore the effect of IL in detoxification can be partially reflected by GST induction.

For studies on the hypoglycemic effect of IL and SEF, only the highest dose of IL (3g/kg) and SEF (100mg/kg) reduced the serum glucose level in the oral glucose tolerance test. However there is no effect on streptozotocin-induced diabetic mice after administration of IL or SEF. It indicated that the acute hypoglycemic effect of IL

and SEF might be related to gastrointestinal glucose absorption. Besides both IL and SEF reduced the serum glucose level. IL also contains about 20% saponin. The hypoglycemic effect of IL may be related to the presence of saponin. Some saponins were found to inhibit active transport by increasing the general permeability of enterocytes in the gut (Johnson *et al.*, 1986). This phenomenon of saponin may be one of the reasons that can be used to explain the effects of IL and SEF on oral glucose tolerance test. The other reason might be related to interaction between saponin and the cell membrane. The interaction of saponin and the cell membrane may cause structural lesions, increasing the turnover rate of intestinal, mucosal cells and resulting in increased nutrient loss.

Both IL and SEF reduced serum glucose level in streptozotocin-induced diabetic mice after administration for one month. It indicated that the chronic hypoglycemic effect of IL and SEF might not only be due to its effect on glucose absorption. According to the result of Glombitza *et al.* (1994), saponin induced pancreatic cAMP level in diabetic animals after one-month administration. An elevated level of pancreatic cAMP may induce insulin secretion. It may be one of the reasons for the effect of IL and SEF.

In the studies on the hyperlipidemic effect of IL and SEF, only the high dose of IL (3g/kg) and SEF reduced serum level of cholesterol and triglycerides significantly.

It indicated that IL and SEF might affect cholesterol and triglycerides absorption. IL and SEF also reduced serum levels of cholesterol and triglycerides in hyperlipidemic mice. The hypolipidemic effect of IL and SEF might be related to the presence of saponin. Saponin can lower serum level of cholesterol and triglycerides in many different studies (Morehouse *et al.*, 1999). It can provide some information for explaining the result of IL and SEF in hyperlipidemic mice. First of all, saponin may interact with cholesterol to form a large complex to prevent cholesterol absorption. Then saponin may also interact with bile acids to interfere with bile acid re-absorption and increase bile acid excretion. It results in increasing cholesterol utilization to compensate for the loss. The final possible mechanism for the hypolipidemic effect of saponin is that it may interact with the membrane of intestinal mucosal cells and result in a change in the permeability of membrane to prevent cholesterol absorption.

IL and SEF also lowered the serum level of cholesterol and triglycerides in diabetic mice. The mechanism may also be related to the presence of saponin and can be explained by the above mechanisms. The hyperlipidemic effect in mice is due to insulin deficiency. Besides, as mentioned in Chapter 3, the hypolipidemic effect of IL and SEF may be related to the induction of insulin secretion.

According to the above results, IL and SEF lowered serum levels of glucose, cholesterol and triglycerides but did not produce any liver cell damage. IL is rich in

saponins. Therefore the effect of IL may be related to the presence of saponin. Besides IL also induced GST activity. However GST induction of IL may be related to the other components in IL. According to the above results, one beneficial effect of IL may be related to prevention of nutrient absorption. Administration of IL before a meal may reduce nutrient absorption and satiate the appetite simultaneously.

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